Introduction to VIROLOGY

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To

FB and FGD

E, B, and TM

RG, LCS, AMS, and GMS

PREFACE

During the past 20 years knowledge of the virus diseases has grown at a rapidly increasing tempo Perhaps the declining importance of bacterial infections has been a spur The invention of new technics and tools has provided new opportunities All sides of the problem have been attacked, pathogenesis, etiology, host resistance and susceptibility, the natural history of virus diseases, and their connections with environment The advance has been typical of our times in two respects, in the debt it owes to modern technology and instrumentation and to a broadened concept of disease that has come from studies of the distribution and mass occurrence of infection geographic and statistical pathology

As a result, our understanding of the ancient virus diseases has deepened new virus diseases have been discovered, new relationships and new biologic phenomena Therapy still lags but prevention has taken several long strides The advance has followed a definite pattern and we now perhaps have enough experience to guide us more directly in the future It is a time of confidence, a confidence tempered by the expectation that our parasitic foes may confound us at times in the future as in the past but that our helplessness will never be as great as it was 25 years ago

It may well be that the stage is set for clinicians and laboratory workers in many hospitals to investigate more thoroughly the virus diseases they encounter The methods are relatively simple, the major hazards well charted A critical bacteriologist with a little additional equipment and a small isolation unit should be able to undertake diagnostic virology and the study of local problems with complete propriety

This book is intended as an introduction to the subject It is grossly incomplete in many respects but may provide a panorama of the problems we face in the States as the author sees them The technical section is intended to introduce bacteriologists and pathologists to the basic virus procedures with emphasis on precautions in handling highly infectious agents and the interpretation of results The book as a whole is based on lectures and laboratory exercises that have been given for some years to students in the School of Medicine, University of Buffalo

Many virologists believe that hospital laboratories should limit themselves to serologic (complement fixation) tests of virus infection, that the isolation of viruses and experimental virology belong only in research institutions This may be good advice in a general way but has two serious drawbacks Certain virus infections are best diagnosed by isolation of the etiologic agent, serologic tests being cumbersome and impractical Secondly, the unusual and instructive cases are quite as likely to occur in one hospital as the next, outside as well as within medical centers In any case, the author could not agree in good conscience, having undertaken virus studies in a hospital laboratory and with no guidance other than a second hand set of Roscoe Hyde's laboratory exercises and one agate mortar and pestle It is hoped that the present suggestions have benefited from the advances made since that time and that the beginner follow his nose but keep one eye on the character of the diseases he may investigate

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INTRODUCTION TO VIROLOGY

Boswell *But of what use will my book be, Sir?*
Johnson *Never mind the use—do it!*

I

THE NATURE OF VIRUSES AND VIRUS DISEASES

THE STUDY of virus diseases has become the liveliest and most productive branch of microbiology during recent years It is already a large field that encompasses a wide variety of diseases and disease producing agents There are reasons for believing it will continue to grow, that additional diseases will be recognized as virus infections Many workers expect viruses may some day be incriminated as causative agents of human as well as lower animal neoplasms and that some lesions we ascribe to degeneration or hereditary factors may be due to unsuspected virus infections Moreover, we may anticipate in the future to discover and describe the viruses responsible for various infectious diseases, the causative agents of which have not thus far been found

The word virus means poison Usage has limited its meaning to infectious poisons and lately to infectious poisons of very small size In the early days of bacteriology all microscopic and submicroscopic agents of disease were called viruses When it was discovered that some were small enough to pass through earthenware filters, they were distinguished as filtrable viruses Now they are generally called simply viruses Earthenware filters were originally used in support of the theory of biogenesis to secure evidence that living agents of disease do not arise from the environment but only from living predecessors The filter that retained bacteria also retained the power to infect Such demonstrations were frequently made in support of the views of Pasteur Yet even in those early days of microbiology it was at times noted that bacteriologically sterile filtrates were nevertheless capable of inciting disease The first to do so was Iwanowski who observed that the juices of plants infected with tobacco mosaic remained infectious after passing through fine filters Five years later Loeffler and Frosch discovered the same is true of the agent of foot and mouth disease These observations did not seriously disturb bacteriologic thought because the filtrates were in any case sterile by bacteriologic criteria, they did not contain organisms that could be cultivated in sterile media

But they were the beginning of virology which slowly and inconspicuously developed in the shadow of bacteriology until some twenty years ago when it began to grow very rapidly In commemoration of the great debate regarding biogenesis or heterogenesis and the part that filters played in the identification of viruses, it is worth noting that the viruses themselves have reopened that old issue There now are those who consider the viruses to be midget microbes and others who consider them unique chemical compounds capable of replication when incorporated into the life of cells The evidence for the second view comes from experience with plant viruses which can be purified and crystallized to a degree wholly satisfactory to chemists and which, after chemical manipulation, are still capable of inducing the phenomena of growth and infection in plants Nothing comparable has so far been demonstrated in the case of animal viruses which seem to be a rather different order of things, but the ancient dispute about spontaneous generation has been given a fresh and unexpected rebirth

Size is therefore a primary characteristic of viruses They are very small They are sufficiently smaller than bacteria, which we measure in microns, that their dimensions are customarily expressed in millimicrons Most animal viruses lie between 10 and 400 mμ or 0 01 and 0 4 microns The viruses thus bridge the gap between bacteria and large protein molecules and complete a biologic scale, evidence, perhaps, that no other group or lower order of infectious agents remains to be found

A more important characteristic of viruses is that they multiply and manifest themselves only in cells, in susceptible living cells This is quite as true of plant as animal viruses It is true of the larger viruses that approximate in size the smaller bacteria They are autocatalytic They are composed chiefly of nucleoprotein with small amounts of lipide and carbohydrate The larger viruses seem to be more complex chemically than the smaller ones but since we are still too clumsy to purify completely the animal viruses, we cannot be sure

Obviously their relationship to cells is of outstanding importance to our understanding of them Some things are known We know a little about how some of them combine with cells Influenza virus, for example, reacts specifically with a focal structure of the cell

surface and destroys it (the receptor) probably through an enzyme As proof heated virus can be shown to combine with the receptor but not destroy it Very few viruses behave as influenza virus does and we know nothing of how most of them combine with their host, but the elegant studies of influenza virus have set a pattern of thought that has been helpful in understanding the relationship between virus and cell Some plant viruses seem to be incapable of invading or attach ing themselves to cells Infection requires the delicate inoculation into the cell that only particular insects are capable of making

Still less is known of the later phenomena of cellular infection although the bacterial viruses have yielded fascinating evidence of a unique kind of intracellular multiplication Shortly after bacterial viruses penetrate the cell membrane an increase of nucleoprotein occurs due only in part to increase in virus The virus body temporarily disappears from sight This stage is followed by the appear ance of 50 to 100 minute particles that then group to form a collec tion of new adult virus forms It is a form of multiplication that entails recombination of diffused elements and that facilitates varia tion Thus a cell infected with different strains of a bacterial virus can hatch hybrid descendants in numbers and proportions predict able on the basis of chance recombination Something similar is believed to be true of influenza virus although the evidence is less complete

One might say that a virus is incomplete in a biologic sense in the absence of a cell Thus the purified virus we see in the electron microscope is not necessarily morphologically complete for in the form we see it outside the cell it is biologically incomplete In the cell its structure may be augmented by borrowed cellular com ponents Only the study of infected cells can solve this old problem We know of incomplete viruses in another sense for certain viruses exist at times in a form antigenically typical but unable to infect or induce disease Certain viruses also occur as components of different sizes the smaller as a rule being incapable of inciting disease

The obligate parasitism of viruses is of practical and theoretical importance Since the virus can only be demonstrated through its effects on cells viruses are shown to exist only through the infection of animals or their tissues in culture The use of living animals com plicates the study of viruses and at times leads to error The experi

mental animals may harbor native unsuspected viruses that are stimulated to cause disease by the inoculation of test material The animals may sicken and die not as a result of inoculated infection but of activated latent infection, often to the complete confusion of the experimenter A notorious example is the occurrence of herpetic encephalitis in domestic rabbits following intracerebral inoculation In this case latent herpes virus infection should be presumed to be the cause until proven otherwise

Their intracellular location shields the viruses from humoral anti bodies Treatment (but not necessarily prophylaxis) by immune serum fails, the virus is at risk only when it is liberated and in process of infecting a new host cell The prolonged immunity to certain virus infections may be due to this mechanism The virus slumbers for long periods within cells and only at times, through the death of a cell is liberated to stimulate antibody formation and reinfect a few contiguous cells Something of the kind may be true of yellow fever and herpes

Because viruses take part in the life of cells, it is not surprising that they are frequently very particular in the kinds of cells they parasitize A virus that causes oral warts in dogs has been mentioned as an extreme example It infects only the epithelial cells of dogs and these only on the oral, mucosal epithelial junction Their tropisms determine host range, pathogenesis, and isolation technics Of recent years the tropisms of many viruses have been modified by laboratory manipulation and these experimental adaptations have made possible important practical advances not only because the viruses could then be worked with more easily but because, with adaptation, they have often lost their pathogenicity for man and become safe and effective vaccines Alternate passage between the susceptible, natural and the resistant, new host, alteration by prolonged transfer in the laboratory, the use of a different experimental animal, and the dis covery of labile strains have all at times provided usefully modified viruses It should perhaps be noted in this connection that most isola tions of human viruses in experimental animals entail some degree of adaptation This is shown by a rapid change in pathogenicity for the new host and increasing titer of infectivity

While some viruses grow extremely rapidly and quickly kill their host cells, others are known to form a benign and enduring partner-

ship The latter account for latent infections Possibly they explain certain tumors It has been postulated that cancer cells may be such an expression of host parasite relationship the virus becoming an integral inherited feature of the abnormal the cancer cell

Because viruses live within cells the most characteristic morphologic effects of infection are within cells The best known lesions are the inclusion bodies which range from the coalesced virus colonies characteristic of variola to the insignificant Group-B inclusions common to many virus infections The electron microscope is now widening our knowledge of the internal morphology of animal cells and promises to open a new field of micromorphology of which the effects of virus infection may be a fascinating part Something is already known of the responses of cells to different viruses and there is promise that characteristic structural changes may eventually be associated with most virus infections

The rapid development of virology owes much to three technical discoveries made during the 1930 s The first of these was the use of the chicken embryo for the cultivation of viruses The original methods, devised by Woodruff and Goodpasture for the growth of pox viruses employed the vascular membranes of the egg Subsequently the egg cavities have been more commonly used the technics have been simplified and the use of the fertile egg extended to many viruses The fertile egg is our least expensive experimental host is free of confusing latent infections and provides a large harvest of many viruses

The second noteworthy advance was the invention of the ultracentrifuge and its application to virology The early air turbine models are currently giving way to electrically driven types The ultracentrifuge permits the concentration of viruses and estimations of their purity and the mass of individual particles Smaller and intermediate models are now freely available and greatly expedite experimental work

The third advance the invention of the electron microscope, followed the design of electromagnetic condensers capable of focusing an electron beam suitable for microscopy While the visualization of viruses has in itself been of interest and value, the future application of the instrument to the study of virus infection promises to be still more instructive

The use of filters has become less important, possibly because there has been little improvement in them In addition to the earth enware and diatomaceous earth filters, asbestos pads of various types and collodion membranes have been used The latter type was exhaustively studied by Elford who determined standard meth ods for their preparation and formulas for calculating their mean pore size and for estimating the diameter of virus particles that passed through them Surprisingly accurate and reproducible meas urements are possible by means of collodion filters but it is becom ing evident that Elford s formula for estimating the size of virus particles does not hold for the smaller viruses All of the filters cur rently available share the disadvantage that a considerable part of the virus activity is retained by the filter and thus lost

The viruses are as a class more stable and resistant to physical agents than bacteria The smaller viruses, with the exception of that of yellow fever, are especially hardy Poliomyelitis virus with stands treatment with cresol, ether, a wide range of pH, lasts in definitely in 50 per cent glycerol, and is relatively resistant to heat Possibly their stability as well as their intracellular location con tributes to the ineffectiveness of therapy Many drugs that modify the metabolism of cells depress the growth of viruses within them But the effect is limited and no practical therapeutic benefit has been demonstrated thus far Control of virus diseases currently de- pends on the prevention of infection, active immunization, and the use of antisera under special circumstances

A considerable number of virus infections interfere with one an other to such an extent that the one alters the course of the other Interferences also occur between bacterial and virus diseases but not so strikingly as between certain virus infections In terms of the simplest virus-cell relationships, combination of virus particle with the cell wall, mutual exclusion is easily visualized Enough is known however, to make it likely that there are other antagonisms be tween viruses than competition for cell receptors Indeed some viruses are thought to intensify the effects of others The interfer ing action of one virus on infection by another may be of epidemio logic importance In the laboratory it is at times confused with cross protection or cross immunity Interfering viruses are anti

genically different The immunity that one induces to another is temporary in nature

We do not have a satisfactory and accepted classification of virus diseases or a sound virus nomenclature Most virologists feel it is premature to attempt such a classification Since this book is intended to be as practical as possible, the virus diseases have been grouped according to symptoms of infection Whether better understanding of the micromorphology of the virus diseases will provide a suitable basic classification of them or more knowledge of the viruses themselves will permit a reasonable grouping cannot be foretold It is hoped that a clinical classification may facilitate the study of human illnesses

II

THE POXES AND THE RASHES

THE HISTORY of the pox, that is, the smallpox—syphilis was once called the great pox—is a fascinating story Smallpox was the first of the plagues to become controllable Jenner's ' vaccination is a landmark in the history of medicine The laboratory study of smallpox broke fresh ground, too Goodpasture introduced modern egg technics in his investigation of pox virus and Parker and Nye in 1925 used vaccinia virus in their pioneer tissue culture experiments The pox viruses are sufficiently well studied and understood to be successfully classified Twenty years ago Goodpasture proposed the nomenclature that is now widely accepted He wrote ' Owing to the fact that the virus class is so difficult of definition, it has seemed to me of primary importance to correlate any small group of virus diseases as specifically as possible whenever the facts warrant such an association " He proposed a genus *Borreliota* Borrel discovered 50 years ago the specific granules of fowl pox The suffix Goodpasture proposed was formed from the Greek letter iota the smallest of the letters, the term meaning the *little particles of Borrel* Within the genus were included four subspecies, *variolae* (the true poxes of animals of which eight are known), *mollusci*, the agent of molluscum contagiosum, *Borreliota myxomatum*, the virus of rabbit myxomatosis, and the agent of fowl pox *Borreliota avium* It is worth noting that the classification is largely based on the characteristics of the pathogenic agents The particles, or elementary bodies, react characteristically to stains and are quite uniform in size and shape, being brick like bodies in the range of 250 to 300 mμ in their longest dimension Interestingly, other virus particles of similar appearance are the causative agents of chicken pox and herpes zoster but these are smaller (200 to 220 mμ) One is reminded of the observation of Jacob Henle, long before modern microbiology was established, that like agents cause like diseases Both the similarities and differences are important and the differences are reflected in the lesions which in the case of chickenpox and herpes, are characterized by intranuclear inclusion bodies while the pox viruses induce intracytoplasmic inclusions The gross lesions

of the two also differ, being more pustular in the case of the poxes

The subspecies are not antigenically related but relationships exist within the variolae as well as differences and these are borne out in minor differences in inclusion bodies lesions and of course, host range and pathogenicity Recent investigations have provided substantial evidence on all these points including small but in teresting differences between cowpox and vaccinia virus Whether the strain of vaccinia being used in the immunization of humans has been altered by long maintenance in the laboratory or was dif ferent when isolated by Jenner is not known Indeed it is impossible to establish that our vaccinia strains are derived from their historic prototypes We would like to think they are

Smallpox virus is so infrequently the cause of disease in the United States that one might assume it is of trivial importance to the hos pital virologist This is true as a practical matter but it remains a useful and instructive agent for experimentation and our knowledge of the disease it causes has been so illuminating it should not be dis regarded Moreover, we must all be always alert to the occurrence of smallpox which is frequently introduced to this country and could become so serious a problem if disregarded

Smallpox is not only one of the most important, longest known and serious of the virus diseases it also illustrates certain features of virus diseases as a class and important principles in the conquest of a virus disease through medical observation and discovery The story of smallpox is largely the story of Edward Jenner who one hundred and fifty years ago introduced vaccination the prophylaxis we still practice Primitive peoples had learned of the immunity con ferred by one attack of smallpox The Chinese are said to have in sufflated dried virus into their noses as an immunizing procedure, and smallpox crusts have been sold on the market places in various parts of the world for a great many years The method of immuniza tion by the direct inoculation of smallpox virus into the skin, vario lation, was introduced into Great Britain in 1718 by Lady Mary Wortley Montague and was successfully practiced until Jenner s observation of the efficacy of vaccination Vaccination substituted *a modified (cowpox) virus for that of smallpox It had been noted* in rural areas of England that an experience with cowpox appeared to confer immunity against smallpox Jenner made of the observa

tion a scientific medical procedure His work is of outstanding significance because he established standards of experimentation that have seldom been excelled Jenner's opportunity arose from the practice, current in England at the time, of variolation Individuals underwent dermal inoculation with smallpox virus as protection against systemic infection The practice had the serious disadvantage that each variolated individual was a source of smallpox infection and secondary cases sometimes followed Indeed variolation was commonly practiced in secret because it caused so much alarm Jenner took advantage of the situation to determine that patients who had been vaccinated became immune to variolation and he methodically tested and verified that fact before recommending vaccination as a smallpox preventive 'I will build a rock," he said, and continued his human experimentation until the evidence was incontrovertible It has withstood the test of the 150 years since Jenner's time with little or no modification

Smallpox was the great plague of the American Indian Vaccination was introduced in North America by Benjamin Waterhouse who had been encouraged by Jenner and who, in turn, instructed Thomas Jefferson Thomas Jefferson is said to have personally vaccinated Indians while he was President of the United States (1801) The control of variola by vaccination remains an essential prophylactic and should be universally practiced since smallpox is a continuing menace that may only be met by the protection of individuals The smallpox rate in Mexico, for example, has in recent times been as high as 150 cases per 100,000 population Smallpox is periodically and not infrequently introduced from foreign countries New York City, in 1947, undertook the mass vaccination of a large segment of its population when a single florid case of smallpox was followed by secondary cases Smallpox was introduced into England on 15 separate occasions in 1946 from Asiatic countries, largely through sailors Paris had similar experiences at the same time which are presumably no more than representative of the periodic threats that are represented by the existence of large areas of infection in many parts of the world and against which our single protection is that of mass vaccination Russia has provided the most recent evidence of the effectiveness of vaccination since vaccination was infrequently practiced there previous to 1919 It has been estimated

that during the last 100 years 5 000 000 persons died of smallpox in Russia and that the institution of compulsory vaccination during the past 15 years has prevented the development of more than 1 000 000 cases, preserving more than a quarter of a million human lives

The variola virus is a brick like body approximately 200 to 250 by 300 to 320 mμ in size It was first described in 1887 by Buist and may be seen in good preparations with a light microscope Paschen s description attracted more attention and the elementary bodies, which are indeed the virus particles, are frequently called Paschen bodies In the infected cell they are seen in colony formations (the cytoplasmic inclusions) of considerable size which are known as Guarnieri bodies The virus is very stable This has been recognized for hundreds of years and made possible the early practice of variolation and vaccination since it was possible to preserve infectivity by storing dried crusts from variola or vaccinia lesions Crusts remain infectious if kept in a stoppered bottle for a year at room tempera ture and may be kept for much longer periods in the refrigerator Frozen, infected allantoic membranes were found infectious after 15 years. The vesicle fluid itself remains active for a month when ex posed to daylight and for nearly three months in the dark Cowpox virus on one occasion was found infective after storage in the re frigerator for seven years and crusts stored *in vacuo* were infective after two years This is of course of great practical importance in the spread of smallpox since infected clothing bedclothes and other fomites represent a serious hazard The virus is killed when heated at 55°C for 30 minutes

The natural history of smallpox and its pathogenesis make a coherent account Following a rather long incubation period (12 days) constitutional symptoms develop which persist for some days and are then followed by the characteristic skin eruption This sequence of events is due to the pathogenesis of the disease which commences with a primary lesion that in man is frequently of the respiratory organs and thus implicates air borne infection as a common mode of transmission, followed by multiplication of the agent within the body and by spread through the blood until a general systemic infection ensues Virus may therefore at appropri ate periods in the early stages of smallpox be readily demonstrated

in the blood stream The virus is there, however, only during the first few days of illness and has been found as a rule in the more serious cases The primary lesion in the mouth or throat is a potential source of infection by means of droplets and the pox lesions of the skin are a continuous menace, as has been stated earlier

Clinical recognition of smallpox is doubtless quite simple if two important practical reservations are taken into account The first of these is that the disease is so seldom seen in many communities that physicians have little or no experience with it and are less disposed to consider the possibility in arriving at a diagnosis The second reservation depends on the fact that the disease in the vaccinated that is, the partially immune, presents particular difficulties It is also of considerable epidemiologic importance since the atypical cases may be quite as infectious as the typical ones This form of the disease introduced into an area in which smallpox is rarely seen constitutes a trying problem It is believed that as immunity wanes following vaccination the persistence of an immunity to the typical skin lesion lasts longer than systemic immunity and that individuals exposed at such times may experience a brief, febrile illness (variola sine eruptione) In some individuals the disease is modified by a low degree of persistent immunity to such an extent that only a few atypical pox lesions occur, lesions which may easily be confused with mild chickenpox or acne It should also be realized that extremely severe cases are in themselves atypical Such cases may be predominantly toxic or hemorrhagic in nature and the patient at times dies before the appearance of skin lesions This is particularly true among the elderly In this case the menace is not so great as in the milder forms of atypical smallpox because the patient is sick enough to be confined to his bed The mild ambulatory case is a greater threat

The onset of symptoms in smallpox is usually abrupt with chills prostration, backache and fever The pains are at times so severe that patients are tempted to throw themselves from the window These manifestations continue for several days and are characteristic only in the presence of other cases They are followed, once the temperature falls, by the rash which begins as discrete papules on the face but rapidly spreads to the extremities and trunk It involves the palms of the hands and the soles of the feet as well as the mouth and pharynx The lesions shortly become vesicular and by the tenth

day, pustular The evolution of the pox reflects the immunologic status of the patient and in the early days of vaccination this phe nomenon was relied on to test the genuineness of skin reactions Thus the practice was common of revaccinating If the second lesion was sufficiently accelerated to catch up and mature with the first, it was assumed that both were due to vaccinia virus and not pyogenic bacteria

The essential lesion, that is, the dermal lesion also occurs in the esophagus and other portions of the gastrointestinal tract It consists of liquefaction and balloon degeneration of epithelial cells the former leading to multilocular vesicles and the latter to giant cells Intranuclear inclusions occur in cells of the rete Malpighii and eosinophilic Guarnieri bodies form in the cytoplasm These as has been mentioned, are in fact colonies of variola virus

While clinical diagnosis is eminently satisfactory under circum stances in which the disease is commonly experienced it is quite evident that the atypical cases which predominate in western coun tries represent a difficult problem Fortunately, laboratory diagnosis of smallpox has become very satisfactory during recent years The fluid from the vesicle for example can be used directly as antigen in a complement fixation test Infectivity may be transferred to the cornea of a rabbit (Paul's test) The most satisfactory laboratory procedure at present is the inoculation of the chorioallantoic mem brane of chicken embryos with material from the suspected lesions Identification of the agent is then made by histologic study of the membrane in which the elementary bodies and inclusions may be found Direct histologic examination of smears from suspected skin lesions may be undertaken and in the hands of experienced investi gators is reliable and rapid The elementary bodies are recognized without difficulty by the experienced observer The method is par ticularly effective during the early days of the disease when diagnosis is, of course, of the greatest importance During later stages, tests depending on the development of antibodies are perhaps simpler in some respects and surely more effective since the virus particles may no longer be found with regularity Direct examination is of limited value in distinguishing between variola and varicella in which ele mentary bodies also occur While it is doubtless true that the expert may hazard a presumptive differential diagnosis, largely based on the

profusion of elementary bodies in variola and their scarcity in varicella, it is clear that the similarities between the two are a severe limitation on the method of direct examination Fortunately, the serologic tests now available, in particular complement fixation reactions and the simpler flocculation test, may be relied upon to provide a specific diagnosis although neither will distinguish between vaccinia and variola It is believed that variola may be distinguished from vaccinia in that the latter is capable of serial transfer in rabbits and that vaccinia produces only cytoplasmic inclusions and not nuclear ones under such circumstances Isolation of variola virus by inoculation of the chorioallantoic membrane of fertile hens' eggs is briefly described in the second section of this volume One further method which is clinical in nature has been recently endorsed It was originally described in 1918 by Tieche and consists of producing an allergic skin reaction through the introduction of the variolous material in an individual made allergic by repeated vaccinations The availability of a suitable individual is of course, a serious drawback It is of interest in this regard to note that the term allergy was coined by von Pirquet to describe the accelerated immune reaction in the skin of vaccinated individuals when re-exposed to vaccinia virus

The control of smallpox rests on the effective use of the two obvious methods of recognition of cases and their quarantine and mass immunization through vaccination Many studies have indicated the importance of revaccination Populations exposed to a considerable risk should be revaccinated periodically Thus, in a comprehensive study in the Near East it was seen that recently vaccinated children were with rare exceptions solidly immune to smallpox while adults had relatively little residual immunity Immunization cannot be delayed until the exposure has occurred Under these circumstances it not only does not protect or modify the course of the disease but at times seems to provoke a more severe attack than would otherwise be expected However, mass vaccination or revaccination is recommended as an immediate prophylaxis in the presence of a threatened epidemic It is possible that gamma globulin could serve a useful purpose in exposed individuals The methods of vaccination are generally known In North America, calf lymph is almost universally employed as a source of vaccinia virus although virus grown in tissue culture and in eggs has been found satisfactory by workers elsewhere

There are three types of response to vaccine the primary take, an accelerated take or vaccinoid reaction, and the immune reaction The first is in all respects a typical pox lesion The second is an immune response representing an allergic reaction to the virus It appears rapidly and rushes through its cycle of development and is quite without the constitutional symptoms that frequently accompany primary reactions Since inactive or killed virus can also induce an immune reaction, such a response is not proof of reimmunization It is necessary to know that the vaccine administered is living that is that it is capable of causing primary reactions in nonimmune persons Vaccination should be practiced as superficially as possible, the pressure method being recommended The use of a subcutaneous route of inoculation, at one time recommended as the method of choice for tissue-culture vaccine, has the serious disadvantage that the degree of response and thus the immune status of the individual cannot be gauged

Vaccination does carry a small but definite hazard When 5 000 000 of the residents of New York City were vaccinated in 1947 in a campaign undertaken to protect the City against smallpox that had been introduced from Mexico, postvaccinial encephalitis was reported in 49 individuals However, of the eight fatal cases, none had characteristic lesions post mortem and in four, death was clearly due to other causes Of the patients who survived all but two recovered completely A rather similar experience was reported from Switzerland in 1946 In both cases, the level of immunity in the population seems to have been quite low Encephalomyelitis is also known as a sequela of natural infections with both variola and cowpox

There is no satisfactory treatment for smallpox but it is quite evident in recent reports that the use of antibiotics and the control of secondary infections have frequently benefited many patients The ultimate effect of such treatment on the mortality rate among cases remains to be determined

The experimental virologist will be interested in recent studies by Downie and his associates, which have shown that cowpox virus occurs in nature as a mixed population Downie recognized two types of lesions on chorioallantoic membranes, a common hemorrhagic one which he called "typical' and a discrete white lesion The variants

could be separated by subculturing from isolated lesions and were found to breed true. The experimental virologist will also find in vaccinia an especially effective interfering agent, one capable of modifying the evolution of a variety of other virus diseases. Whether this is in part due to the slow evolution of vaccinia and therefore the prolonged growth of large amounts of virus in many animals is not known.

III

VIRUS DISEASES OF THE LIVER

THREE VIRUS infections characteristically involve the liver and cause jaundice All three are important common diseases we have learned much about within recent years Two of them infectious and serum hepatitis, are world wide in their distribution The third, yellow fever, is at present effectively quarantined in South America and Africa

Infectious hepatitis

Infectious hepatitis is an enteric infection The virus is a denizen of the bowels of patients and carriers Viremia is also present but does not seem to be important in the transmission of infection which commonly occurs by means of fingers and fomites contaminated with fecal virus or through the fouling of water or food The explosive outbreaks, the disease is also called *epidemic hepatitis*, presumably follow failure in sanitation the slowly developing or slumbering epidemics and sporadic cases are more often due to personal contact between infected and susceptible individuals The large epidemics parallel outbreaks of typhoid fever and dysentery and simultaneous epidemics of dysentery and hepatitis have often been encountered

Prevention is blocked chiefly by our inability to identify healthy carriers by the laboratory identification of the virus or of infection In contrast, the typhoid carrier can be identified with certainty Nothing better illustrates the value of laboratory aids in diagnosis in the control of infectious disease than the contrast between our successful control of typhoid fever and our complete failure to suppress infectious hepatitis

Infectious hepatitis is much like poliomyelitis It, too, is chiefly a disease of children Hepatitis occurs more commonly in the autumn and winter months, later than poliomyelitis or the enteric infections, but this is possibly due to the longer incubation period The spread of infection may not be much different seasonally from that of the others Most infections, especially in the very young are subclinical, nonicteric, just as most poliomyelitis infections are nonparalytic.

Only the occasional icteric patient may indicate the nature of an epidemic Both infections seem to be primarily enteric and both are thought to be most prevalent in the tropics where early immunization reduces the frequency of sickness *Epidemics* of clinical hepatitis and poliomyelitis are less frequent in the tropics than elsewhere because infection, at an early age, is more common Similarly, both diseases show a higher morbidity in the country than in cities and the lowest rates in the largest cities, all presumably because of the level of subclinical infection during infancy Clinical disease in both cases confers permanent immunity Both infections are world wide It is possible that hepatitis, like poliomyelitis, is showing a shift in age incidence for of recent years epidemics are being encountered in older people than formerly

Infectious hepatitis masqueraded for years behind the misleading name catarrhal jaundice Being benign, there were few opportunities to examine cases post mortem and it was postulated that the jaundice was obstructive, due to occlusion of the ampulla of Vater by swelling of the mucosa or plugging of the lumen The first clue to the true nature of the disease came from an observation of Eppinger during the First World War He had an opportunity to examine a number of battle casualties who had been convalescing from "catarrhal jaundice" when killed, and found patches of hepatitis Eppinger's brief note attracted little attention but during the Second World War his observation was repeatedly confirmed and Rich not only verified the presence of a necrotizing hepatitis but excluded lesions of the ampulla The disease, infectious hepatitis, is unrelated to lesions of the bile ducts

It has since been proven that infectious hepatitis is caused by a virus The proof consists of the experimental transmission of the clinical disease to human volunteers by the oral or parenteral inoculation of feces or blood of patients An experimental host has not been found although all of the common laboratory animals and a variety of exotic ones have been tested The human experiments leave no doubt regarding the nature of the agent but, being necessarily limited in numbers and man being an unsatisfactory experimental animal in many other respects as well, they have left much unsettled Most recently Henle and his colleagues cultivated an agent in liver cell tissue cultures and later in eggs that induced some

of the symptoms of hepatitis in volunteers Evidence was also secured of active immunity to challenge and of the development of an immune skin reaction to ultraviolet irradiated egg material containing the agent Unfortunately these promising results have not been confirmed or extended and we still do not have a laboratory adapted strain of hepatitis virus or certain proof of its cultivation outside of man

The virus is, of course, resistant to intestinal ferments It survives heating at 56°C for at least 30 minutes It is stable in the cold for long periods and is somewhat resistant to chlorine Infectious water that has been properly coagulated and filtered is inactivated if the chlorine concentration (free chlorine) reaches 1 part per million In untreated, cold water the agent probably survives for very long periods of time Olin described a Swedish epidemic in which lake water seems to have remained infectious for months Infected wells have been recognized by others

The persistence of virus in the feces after infection is thought to be quite variable but the number of observations is very small A number of instances have been observed in which carriage lasted for more than a year although we might question whether infectious or serum hepatitis was present The *incubation* period varies from three to five weeks In experimental infections it has usually been three weeks

The clinical signs of infection are anorexia and nausea, at times with vomiting, a slowly enlarging liver with abnormal liver function, slight fever, and then, in some cases, the occurrence of jaundice with symptoms of depression, clay-colored stools, and dark urine The spleen is frequently enlarged as are the posterior cervical lymph nodes Jaundice may be the first sign It is estimated that 5 per cent of all cases become chronic Relapses are not infrequent All of these manifestations have varied widely at different times and places

Serum and infectious hepatitis are clinically indistinguishable but the study of outbreaks has suggested that there are minor differences between them Serum hepatitis is more likely to begin insidiously with little or no fever or gastric symptoms during the preicteric stage Infectious hepatitis begins more abruptly, usually with a degree or two of fever

A fulminating form of infectious hepatitis has occurred at times

among military personnel In these outbreaks among young adults, the prodromes have lasted for but two or three days and death frequently ensued without jaundice Outbreaks have also been described, as in Denmark, in which the course of the disease has steadily progressed to death in as many as two-thirds of all hospitalized patients The Danish epidemics have chiefly affected women more than 40 years of age The unusual severity of hepatitis in elderly women has been noted elsewhere, the unusual circumstances that predisposed to the epidemic among women in Denmark are wholly unknown

Possibly one per cent of all cases of clinical jaundice proceed to chronic hepatitic cirrhosis Many individuals have been studied by repeated liver biopsy and the sequences in the liver carefully documented The relative importance of the virus of infectious hepatitis in the causation of cirrhosis is not known but there seems no reason to doubt that it is one of the causes

The peripheral blood early shows a moderate leucopenia Later in the disease abnormal mononuclear cells that simulate the Downey cells of infectious mononucleosis make their appearance Cases of mononucleosis with jaundice and hepatitis with mononucleosis are reported from time to time and raise the question whether the two diseases are related Until precise etiologic diagnosis is possible, this and many other basic questions cannot be answered Myelitis and meningitis have complicated infectious hepatitis at times and there is some evidence that infection during the first trimester of pregnancy may damage the embryo and cause congenital defects

The hepatic lesion consists of a necrosis of the liver cells that begins in the center of the lobule Regeneration, variation in cell size, mitotic figures, and various stages of degeneration may all be seen Some degenerate cells appear to be pushed out from the cords become rounded and resemble the Councilman bodies of yellow fever

Diagnosis rests heavily on clinical observation but is somewhat aided by liver function tests The thymol turbidity and cephalin cholesterol flocculation tests are useful Bilirubinuria reflects hepatic damage of the kind that occurs in infectious hepatitis All of these have value in anticipating the occurrence of jaundice and in judging when a patient should be discharged from the hospital They are also useful in identifying subclinical cases during an epidemic There are

no specific diagnostic procedures Effective temporary immunity can be provided exposed individuals by the intramuscular injection of human gamma globulin (0 01 ml per pound of body weight) Passive immunity is effective up to one week before the onset of symptoms Treatment consists of the judicious use of rest and a high caloric diet low in animal fat but rich in protein

Serum hepatitis

Serum hepatitis, a disease of growing importance, is clinically in distinguishable from infectious hepatitis but is transmitted ex clusively by the injection of or contamination with the infected blood of patients or carriers Its origin is a mystery In its present form, it seems to be a wholly artificial disease, one that could not exist with out the practice of parenteral therapy that was created with or by the hypodermic needle The virus of serum hepatitis has never been demonstrated in patient s feces Our epidemics are all man made

The meager differences between infectious and serum hepatitis have been mentioned There is one more striking difference, the incubation period Serum hepatitis has a prolonged incubation, from two to five months Aycock trying to match this with the unnatural transmission suggested that the long incubation might be due to the fact that infection is by means of serum virus mixtures, that the serum contains antibody and that the infecting inoculum is therefore an incompletely neutralized dose of virus Prolonged incubation is characteristic of serum virus mixtures tested experimentally Ay cock's supposition was that the viruses of serum and infectious hepa titis are identical the one conspicuous difference in the diseases being due to the mode of transmission Since then some evidence has ac cumulated that the two viruses differ antigenically but the issue is by no means settled

Dutch observers report that in Indonesia where infectious hepa titis virus infection is presumably prevalent native troops are more resistant than Dutch soldiers to serum hepatitis as shown by their relative freedom from syringe jaundice They appear to be immune to both viruses This was interpreted to mean that the two cross protect since the native population had had no previous experience with serum hepatitis virus The origin and nature of the virus of serum hepatitis can hardly be determined from such circumstantial

evidence At present we simply do not know whether we are dealing with one or several viruses

The two common ways of contracting serum jaundice are contaminated syringes and the use of pooled plasma in the treatment of shock and burns Of lesser frequency are infections acquired through the handling of contaminated blood by laboratory technicians, physicians, and nurses The frequency of infection following the administration of plasma, especially pooled plasma, has at times been very high In recent years more than 10 per cent of all recipients of pooled plasma have been found to develop serum hepatitis Since the incubation period is often very long and the onset remote from the injection of the plasma, the connection was unsuspected until plasma was so extensively used during the Second World War On the basis of data accumulated then it has been estimated that 0 35 per cent of donors are infected At present, plasma is being irradiated with ultra violet light, an early and limited trial having suggested that such treatment inactivates the virus of serum hepatitis The evidence is now quite convincing that irradiation at the energy levels so far used is ineffective and many instances are known in which batches of treated plasma have been the cause of disease The use of pooled plasma should be reserved for emergencies in which the immediate need is critical enough to justify the risk of infection Human albumin, as separated by the cold ethanol method, is a safe substitute and will presumably replace plasma in the treatment of shock unless other substitutes or an effectively sterilized plasma is developed that will replace both The danger of infective plasma is multiplied when many plasma samples are combined as was done during World War II If the incidence of serum hepatitis viremia is in the range of 0 25 to 0 5 per cent, it is evident that pools of fifty or a hundred bleedings will frequently be infectious Considerable protection is secured by limiting the size of pools to ten bleedings and by excluding donors with a history of jaundice, with abnormal liver function or those from a community in which infectious jaundice is known to be present The blood of such individuals can safely be used for fractionation

The second form of serum hepatitis was first suspected by Swedish physicians (Flaum, Malmros, and Persson) who noted an epidemic of jaundice among patients attending a diabetes clinic Jaundice had

been accepted in syphilis clinics for years where it was usually ascribed to the action of arsphenamine on the liver We know now that the delayed cases of jaundice are not due to the drug but to needles and syringes contaminated with the blood of hepatitis virus carriers The changing of needles alone does not protect since blood is commonly regurgitated into the hub of the syringe The danger is real and brief boiling of instruments and the inadequate sterilization of Hagedorn needles in alcohol and similar procedures should be replaced by steam sterilization An alternative good practice calls for 10 minutes of active boiling of syringes and needles Chemical disinfectants are unreliable

Serum hepatitis is not contagious, the virus has never been demonstrated in the feces or transferred by oral inoculation In these respects the risk is small The survival and spread of the disease are due to the prolonged persistence of infection (and viremia) and the opportunities of transmission we ourselves provide A number of healthy individuals have been proven to be carriers for years In them the virus apparently survives in an innocent fashion much as herpes virus endures endlessly in its human hosts

Yellow fever

Yellow fever is the third of the hepatotropic viruses Yellow fever is an ancient disease that had struck a biologic balance with its hosts in the wildernesses of South America and Africa a balance that was rudely upset when the infection was transferred to the port cities of the world by the sea traffic of the 18th and 19th centuries We ourselves experienced 95 epidemics of yellow fever before the turn of the century some 500 000 cases occurred in our port cities

The story of the conquest of yellow fever deserves a place in this book for two reasons First of all it was the first great achievement of American medicine Secondly, it teaches much of the nature of an unusually well studied virus disease and, therefore, of virology It inevitably is also the story of Walter Reed who after 18 years as an army surgeon in frontier posts, learned bacteriology from William Welch and became the Army's most distinguished student of infectious diseases When Reed went to Cuba to deal with yellow fever his mind was prepared by the experience of our Spanish War which had taught the importance of the fly in the transmission of typhoid and

the recent discovery of the man mosquito cycle of malaria He also knew that the homes of yellow fever patients did not become in fective for 15 to 20 days, suggesting an intermediate incubation of the agent in a vector

Much of the remaining evidence was close at hand for Carlos Findlay had suspected the role of the mosquito for many years and gladly provided mosquito eggs and advice of great value But it was Reed who transferred Findlay s theories into substantial fact by means of his well conceived and conclusive experiments among human volunteers Reed succeeded in proving not only that mos quitoes transmitted the infection but that direct physical contact with patients does not do so He also established the filtrability—the virus nature—of the agent and determined the incubation period

The control of yellow fever in Cuba and Panama followed directly from Reed s work and for a time it was hoped that if the remaining centers of infection were dealt with the disease might be extermi nated The Rockefeller Foundation undertook to finish the job, at the time considered to be simply a problem of mosquito control and quarantine This effort was frustrated by the observation of epi demics of yellow fever in forested areas of South America in the early 1930 s and the discovery that the disease exists in jungle animals among which it is transmitted by arboreal mosquitoes (and pre-sumably by other unrecognized means as well) Fortunately the Foundation's scientific studies meanwhile taught us much about yellow fever beyond methods of controlling *Aedes aegypti* mos quitoes Monkeys had been found to be susceptible and later the virus was adapted to mice and, finally, to fertile eggs This sequence of events, first, the proof of the nature of the causative agent, sec ondly, the establishment of the disease in the laboratory, and, finally, the cultivation of a modified strain of the virus that affords a safe means of artificial immunization, is a pattern that has dominated the thought of virologists ever since To which might be added the dis covery of an animal reservoir of infection with secondary human epidemics dependent upon a new opportunity for the virus to invade new territories

The symptoms of yellow fever in man appear suddenly or insidi ously after several days of high fever Abdominal pain, at times with vomiting is followed after one to three days by the appearance of deepening jaundice which, in turn, is succeeded by a stage of reac

tion, severe prostration, black vomitus, hemorrhages, and often death

The essential lesion of yellow fever consists of a hepatic necrosis that characteristically commences in the mid zone of the lobule It is a highly characteristic lesion Councilman bodies, formed by degeneration of the cytoplasm of liver cells, rounded and acidophilic in nature, are thought to be pathognomonic of yellow fever They are sometimes extruded from the hepatic cords and engulfed by the Kupffer cells Somewhat similar bodies have been seen in infectious hepatitis, which suggests that more caution may be required in identifying Councilman bodies in the absence of other changes than we have long believed The hepatic lesion of yellow fever is sufficiently distinctive to have played an important part in the study of the disease among native populations for it was early learned that a certain diagnosis could be made from liver fragments taken with a trocar Intranuclear inclusion bodies occur in one-fourth of the cases They are similar to those of Rift Valley fever The intranuclear inclusions are named for Magarinos Torres, a pioneer student of the disease

The virus which is one of the smallest, 15 mμ in diameter, is unusually labile, being quickly destroyed by heat It can be preserved for years in a frozen state (yellow fever vaccine must be similarly handled) or desiccated while frozen Our knowledge began to expand in 1928, when it was shown that the monkey is susceptible, and has multiplied rapidly since Theiler adapted the virus to tissue culture and mice This development led by direct steps to the perfection of a vaccine The adapted Asibi strain, also known as 17D, is used as a living vaccine, having become nonpathogenic for man following prolonged cultivation in the laboratory

Much concern was occasioned during the Second World War when a particular lot of 17D vaccine (Lot 467) caused jaundice in one-fourth of the 300 individuals vaccinated with it It was feared at that time that the vaccine strain had become pathogenic but investigation showed the fault lay in the human serum incorporated in the preparation It contained serum hepatitis virus

Diagnosis during the acute phase may be verified by the inoculation of monkeys with patient's blood. Neutralization and complement fixation tests of paired serum specimens are also widely used and entirely satisfactory procedures Immature mice are much more susceptible to yellow fever virus infection than adults

IV

VIRUS DISEASES OF THE RESPIRATORY TRACT

THE HODGEPODGE of acute contagious respiratory infections that plague us every winter is due to viruses, chief of which are the viruses of influenza, acute respiratory diseases (ARD) or what the British call acute febrile catarrh, and the common cold It is usually impossible to distinguish these symptomatically but influenza and ARD virus infection can be proven by laboratory means The others we lump as common colds, knowing only that a virus (or viruses) is responsible, a virus that has not yet been trained to laboratory use

We have known that there is a common cold virus much longer than we have known of influenza virus but, not having discovered a susceptible laboratory animal or perhaps how to infect laboratory animals, we know very little about it Nothing better illustrates the importance of experimental virology than the contrast between what we know of these two agents It is an old story, the story of yellow fever in the days of Walter Reed, the story of virus hepatitis and hemorrhagic fever today For all that we know of the common cold virus has been learned from human experiments On the other hand, we now can grow and measure influenza virus We know what it looks like We can detect infection and can identify and compare strains, all by relatively simple means

Quite possibly we could learn to distinguish the diseases clinically if we were able to sort them out etiologically The distinguished studies of the Commission on Acute Respiratory Diseases led to the separation of what the Commission called undifferentiated acute respiratory disease from atypical pneumonia, exudative pharyngitis, influenza, and the common cold but in this work the clinical and epidemiologic clues were supported by experimental infection of soldier volunteers Moreover the Commission was able to observe outbreaks as well as individual illnesses The composite character of an outbreak is frequently more revealing than single cases

Influenza

Epidemic influenza is rather characteristic, both clinically and epidemiologically An outbreak provides a reasonable presumptive

diagnosis and, under such conditions, the individual cases become distinctive Thus, while the run of sporadic upper respiratory infections is indistinguishable and nondescript, influenza defines itself both en masse and singly during severe epidemics

The pandemic of 1918 was highly characteristic It swept the world in three great waves of infection that attacked half the earth's population Despite the high morbidity rate, mortality was low, only 3 per cent Yet even this accounted for an estimated half million deaths in North America, 100,000 in New York alone High morbidity, low mortality explosive spread in waves, and irregular periodicity are distinctive features of influenza epidemics Physicians recognized the unique character of the 1918 pandemic and also the peculiarities of the individual illnesses Both features greatly impressed William Welch when he inspected the early outbreak at Camp Devens

The immediate response was an intensive search for the influenza bacillus Pfeiffer had discovered during the 1889 epidemic Pfeiffer assumed the organism was responsible because in his experience, it had only occurred in cases of the disease This was recognized as wholly inadequate proof of an etiologic relationship and, as a matter of fact other bacteriologists had found *Hemophilus influenzae* under quite different circumstances Nevertheless, the assumption survived until put to the decisive test of 1918 when it became evident that the influenza bacillus did not account for the facts Indeed it was relatively uncommon in some outbreaks the frequency of isolation varying from 5 to 90 per cent When the pandemic was spent, it was agreed that *H influenzae* was not the cause that the etiologic agent had not been identified and that it might well be a virus rather than a bacterium

The agent responsible for influenza as we know it (and presumably for the 1918 pandemic as well) was not identified until 15 years later when it was isolated by Smith Andrewes, and Laidlaw Their discovery may have been predetermined in part by Shope's success, in the interim in proving that swine influenza is due to a filtrable agent and to the earlier success of Laidlaw with Dunkin in establishing dog distemper, a similar disease in ferrets In any case, a search was undertaken for an influenza virus and ferrets were chosen as a likely experimental host. Indeed, the first symptoms in their

ferrets, following the inoculation of secretions from human cases of influenza, eventually proved to be an accidental distemper infection rather than influenza but the initial encouragement had mean while prompted other trials which brought success The British workers established the nature of their agent and its relationship to human influenza by demonstrating specific antibodies in the sera of individuals convalescing from influenza, antibodies capable of neu tralizing the virus in ferret experiments

Some time later it was learned that infection could be transferred from ferrets to mice and for a number of years the mouse was the animal of choice Still later, influenza virus was adapted to fertile hens' eggs and it was discovered that eggs are also suitable for the primary isolation of human strains Ferrets are little used at present other than for the preparation of antisera but the mouse is still valuable in the study of the experimental disease The chicken embryo is the animal of choice for the propagation of virus and its titration and has so greatly facilitated the study of influenza virus that it is now perhaps the most thoroughly investigated virus of all

Many virologists suspect, however, that we still know less of the disease than we know of swine influenza which is believed to be a vestige of the great pandemic, the consequence of a spontaneous natural adaption to swine of the pandemic strain For example, we do not know where human influenza virus rests between epidemics Shope showed that swine influenza goes underground, may exist for long periods of time in the larvae of the hog's lungworm which embryonates in the earthworm A pasture can become infected with swine influenza and when, in another year, susceptible animals are placed on it and become parasitized by the lungworm, they also become latently infected with influenza virus Miserable weather and chilling and the presence of *Hemophilus influenzae suis* can induce clinical influenza and, in a herd of susceptibles, infection passes rapidly and directly to other animals and results in an explo sive epidemic Nothing to match this is known of the human disease but there is a suspicion that it, too, may lie dormant in some unsus pected reservoir The alternative theory is that unrecognized sporadic cases maintain the virus between epidemics but the evidence that this is so is inconclusive and disputed The periodic appearance and disappearance of influenza virus, which may hold a key to control, remain a mystery

Influenza virus occurs in two physical forms as a sphere approximately 100 mμ in diameter and as long thread like forms easily seen in a dark field microscope Thread like influenza virus has been but recently discovered and remains a wholly unique and unexplained phenomenon Only freshly isolated strains occur in threads The virus is associated with a toxin that is capable of killing mice within 24 to 48 hours

Both the toxic and infective properties are neutralized by specific antisera. The virus occurs in a seemingly endless number of strains that have been grouped into three types, A B, and C The types are immunologically entirely distinct the strains within them related There is a continuous progressive variation in the antigenic composition of the influenza viruses The original strain WS, for example has vanished Its reported isolations in recent years are likely due to laboratory contamination The degree of change from year to year is variable At times the differences have made stock vaccines ineffective last year s vaccine not protecting against this year s outbreak In other years the changes have been minor

The changeableness of influenza virus in nature is matched by its lability in the laboratory where it has often been altered by manipulation It is therefore quite reasonable to conceive of the pandemics as a consequence of the emergence of an especially dangerous mutant The probability of mutants must be considerable in view of the incredible number of virus particles represented by a single case much more by an epidemic We assume that many mutants are ineffective pathogens but that now and then one arises capable of igniting an epidemic and with an opportunity to do so This is at any rate current theory Only direct observation of a pandemic may be expected to settle the question Indeed we have no *proof* that the 1918 epidemic was due to influenza virus as we know it

Nevertheless there is a good fit between what we know of experimental influenza and the natural history of the epidemic disease Furthermore there is epidemiologic evidence that an abrupt change in the character of the 1918 epidemic actually did occur marked most surely by a sudden change in age distribution The abnormal conditions that existed during World War I may have facilitated or stimulated the epidemic

The symptoms of influenza develop abruptly and rapidly after a very brief incubation period (one to two days) Characteristically

one or a few cases occur in a community followed by hundreds or thousands a day later During severe epidemics the patients become desperately sick within hours, the common symptoms being head ache, rigors, fever, epistaxis, and inflammation of the nose, throat, and bronchi Cyanosis was a notable feature of the pandemic The early symptoms are often followed by signs of pneumonia and pleurisy, a consequence of secondary bacterial infection Most of the deaths during the pandemic should be blamed on these secondary infections in which pneumococci, influenza bacilli, streptococci, and staphylococci played the largest part Presumably the sulfonamides and antibiotics would have protected against this deadly aspect of influenza if they had been available They may indeed be responsible for the rapid decline in mortality from pneumonia and influenza that has taken place during the past 15 years, a reduction of nearly 75 per cent

The lumping of pneumonia and influenza deaths is justified by the intimate association of the two It is believed by some that the perio dicity of pneumonia follows the known periodicity of influenza as though the virus even in mild forms of infection, opens the door for bacterial invasion In this respect we may be dealing with circum stances similar to those that occur in swine influenza

The lesions most characteristic of the pandemic influenza were pleurisy, hemorrhages, and inflammation of the terminal bronchioles and adjacent alveoli The terminal air passages were frequently lined by a thin hyalin membrane which may have accounted for the cyanosis Necrosis and desquamation of the ciliated epithelium of the bronchi and trachea were also noted and these have since been studied experimentally in great detail In ferrets and mice the goblet and columnar cells are quickly destroyed, probably as the initial effect of virus growth Intense regeneration from the basal layers follows Interesting differences in the response of respiratory epithe-lium to primary and secondary attacks have been described In the mouse, abnormalities of the nuclei and cytoplasm have been seen as an initial effect of the virus These intercellular lesions may be still more characteristic and it seems probable that influenza might, with experience, be identified by cytologic study of the infected epithe lium

Since only full blown influenza epidemics are sufficiently character

istic to warrant a presumptive etiologic diagnosis and it is only under such circumstances that the symptoms and signs assume diagnostic significance precise recognition requires the demonstration of the agent or serologic evidence of infection Isolation of virus is too tedious and expensive to lend itself to the routine diagnosis of individual cases and is usually reserved for the isolation of epidemic strains and their characterization Nasal washings or gargling, using nutrient broth or skimmed milk as menstruum are treated with penicillin and streptomycin and inoculated into the amniotic sacs of chicken embryos

There are three useful serologic tests of which the simplest is the demonstration of serum antibodies that prevent influenza virus from agglutinating red blood cells This is the test Hirst developed from the observation that if a blood vessel of a fertile, infected egg is torn during harvest of influenza virus the liberated cells promptly clump The hemagglutination of chicken or human group-O cells by influenza virus provides a simple and direct test of the presence and titer of either virus or antibodies and both may be identified if known virus and antiserum are available Virus reacts quickly with red blood cells the test being read within the hour If the amount of virus is considerable the reaction may occur within a few minutes After several hours the two dissociate the cells having become incapable of further combination The virus on the other hand is still capable of combining with fresh cells

The reaction is specifically inhibited by immune serum and the development of inhibitor parallels the development of neutralizing antibodies Both appear in appreciable titer within the week following infection and reach their maximum concentration in fourteen days The titer of virus is determined by establishing the highest dilution capable of agglutination The titer of inhibitor is measured by testing various dilutions of the serum in a system containing a known amount of virus and cells

Immune serum will also fix complement in the presence of influenza virus antigen For this purpose a soluble component of the virus is used the suspension being freed of elementary bodies by high speed centrifugation The egg membranes are the source of this antigenic fraction The complement fixation test distinguishes between recent infection and vaccination and is especially valuable in surveys

The third test is the classical one of neutralizing virus by mixing it with immune serum and thus preventing infection Neutralization tests may be made in mice, dropping the serum virus mixtures in the mouse's nostrils, or in eggs where the result is measured either by the death of the embryos or the growth of virus in the allantoic fluid as determined by hemagglutination

Serologic tests require a comparison of serum samples collected early and late in the course of the disease or the demonstration of a significantly high titer of antibodies During an epidemic the comparison of a number of sera from early cases with an equal number of sera from healthy members of the same community is often sufficient A rising antibody titer or unusual prevalence of high titered sera is necessary because the presence of antibodies is commonplace and does not fix the time of a previous infection

The viruses of mumps and Newcastle disease of chickens react *with red blood cells much as influenza virus does but at a different* rate It has been suggested that the comparative activity of the three represents qualitative differences, a gradient of hemagglutination but it seems more likely to be a difference in degree rather than kind The reactions may be inhibited by other things than immune serum Egg white, a number of mucins, urine, and normal serum to mention but a few, may inhibit hemagglutination by influenza virus Tests for inhibitors are customarily made with virus partially inactivated by heat and capable of combining but not eluting from red blood cells Weak reactions are thus read more easily Inhibitors combine with virus as do the receptors of blood cells and it has been possible to modify the infectivity of influenza virus by treatment with inhibitors Unfortunately none of these reactions has so far provided prospects of clinical therapy and we have no specific treatment of influenza virus infection

The control of influenza offers better prospects Largely through Army studies, we have learned that vaccines containing concentrated suspensions of killed viruses are effective in preventing and modifying *influenza for a period of some months* Given with mineral oil adjuvants, immunity may perhaps be significantly prolonged At present vaccines are commonly reserved for the protection of special groups, as the military, or individuals so weakened by age or disease as to require special protection In the presence of an epidemic of

unusual severity influenza vaccine would very likely be widely used To use it properly it would be necessary to anticipate the epidemic by some weeks since vaccine given immediately before infection is valueless and possibly harmful The world wide collaboration of investigators interested in influenza, who have been affiliated by the World Health Organization, is intended in part to provide such intelligence as well as to investigate the global epidemiology of this interesting disease The stock vaccines now available frequently cause local and even systemic reactions that at times are as much an inconvenience as the mild forms of influenza Moreover, they contain egg protein (the virus is cultivated in eggs) and attention must be paid to sensitivity and sensitization to egg protein Finally, they have the disadvantage that our next epidemic may be due to a strain of virus sufficiently different immunologically from those used in the manufacture of the vaccine as to reduce the value of the immunization This happened in 1947 and very likely will sometime occur again We should be prepared to quickly produce vaccine from the current epidemic strain This is feasible if the strain is promptly isolated and identified Such a vaccine may be expected to reduce the incidence of influenza by approximately 80 per cent

Influenza is an unfinished story despite the excellent work of the past twenty years The virus has been a dangerous enemy and is so facile and adaptable we must expect it to make trouble again Fortunately we are better prepared than we were in 1918 Meanwhile, more attention might be given to interepidemic periods in the hope of establishing means of control on a more effective basis than transient immunization

The common cold

The common cold has turned the lances of many investigators It is well established that a filtrable agent is the *immediate* cause Kruse successfully infected human volunteers in 1914 and Dochez transmitted the disease to chimpanzees in 1928 In 1936 he reported the transmission of infection through eggs and similar results were reported by other workers in the years that have followed None of the experiments has yielded an established laboratory strain and most have ended in disillusionment Indeed Andrewes, who has conducted extensive common cold experiments, has expressed some

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The common cold

The common cold has turned the lances of many investigators It is well established that a filtrable agent is the *immediate* cause Kruse successfully infected human volunteers in 1914 and Dochez transmitted the disease to chimpanzees in 1928 In 1936 he reported the transmission of infection through eggs and similar results were reported by other workers in the years that have followed None of the experiments has yielded an established laboratory strain and most have ended in disillusionment Indeed Andrewes, who has conducted extensive common cold experiments, has expressed some

doubt that the virus has ever been truly propagated in fertile eggs He confirmed the earlier estimates that the virus is approximately 50 to 60 mμ in diameter and verified its presence in the nasal secretions during the day preceding as well as the day or two following the onset of symptoms These are matters of practical importance since they represent the period of greatest infectivity, a period during which every considerate person should quarantine himself Andrewes was led to suspect that there are many normal carriers of the virus One of the difficulties in his investigation proved to be the resistance of many normal individuals to experimental infections This raised hob with many of his experiments

Some interesting histologic studies have been made of the lesions in the nasal mucosa during infection, which have shown that the changes in the superficial epithelial cells and the manner of regeneration are similar to those in influenza

Andrewes postulates that in large communities the virus of the common cold is constantly being passed from one individual to others, usually without causing symptoms excepting in the occasional person The susceptible individual develops symptoms of a cold and at such a time disseminates the virus in greater quantities and may become the source of other clinical cases of disease In smaller communities the infection dies out Many observations confirm the correctness of this view Isolated communities in Alaska for example, have repeatedly reported the complete absence of colds for long periods of time until the arrival of an outsider, in one instance a shipment of clothing was believed to have reintroduced the virus into the community since its receipt was followed by a small epidemic Not much more is known of the common cold other than its seasonal distribution, similar to that of influenza, that epidemics frequently show increasing severity as they develop and that older people appear to become relatively resistant, whether from immunization or constitutional changes we do not know

It has lately been announced that the virus of the common cold has been successfully grown in human embryonic lung epithelium tissue culture Human embryonic tissues are not easily available but virologists will assume that further adaptations cannot be long in coming and that the tantalizing cold virus will shortly be put through its paces in the laboratory

Acute respiratory disease or febrile catarrh

Epidemiologic and clinical studies have long suggested the existence of more than one variety of cold or, to be more precise, of influenza These observations were significantly extended during World War II by human experiments conducted under the auspices of the United States Army in which young volunteers were infected with nasal washings and garglings from patients The outcome of these trials strongly suggested that acute, undifferentiated respiratory disease (ARD) should be distinguished from the common cold as the epidemiologic studies had indicated, for volunteers convalescent from the former remained susceptible to the cold virus

The disease in question resembles influenza more closely than it does the common cold It may be distinguished from influenza by serologic means and bacteriologic studies indicate that it is not due to streptococcus infection Cough, sore throat and hoarseness are common complaints constitutional symptoms, while of brief duration, are moderately severe Fever persists on an average for three days The volunteer studies further showed that the disease differed from the common cold in the time required for its incubation which was set at five to six days rather than the one or two-day interval required by the common cold

Recently the agent responsible for ARD or febrile catarrh has been successfully cultivated in human cancer cells cultured *in vitro* and found to be related or identical to a virus that has also but recently been isolated from adenoidal tissues Antibody response has been seen to occur promptly and clearly and the early data promise that this further example of acute respiratory virus infection will soon be subject to intensive study

Atypical pneumonia

Atypical pneumonia like aseptic meningitis, is a clinical scrap basket that includes pneumonias due to a variety of agents, many of which are still unrecognized Certain well known viruses may cause atypical pneumonia, among them 'Q' fever, psittacosis, influenza, and measles Infectious mononucleosis may be associated with atypical pneumonia but in this case the virus has not been identified and the connection may have no etiologic importance Apparently unrelated to any of these is a group of cases that has in the past dozen

years been designated as primary atypical pneumonia These also are presumably due to a virus or viruses not yet isolated The term primary atypical pneumonia" was adopted by the Army in 1942 and has replaced the older terms 'influenzal pneumonia" and bronchopneumonia " It is nosologically superior because it is more precise

Primary atypical pneumonia is a minor but at times protracted respiratory infection marked by an often unexpected consolidation of the lung demonstrable by x ray examination The pulmonary lesion has become a prominent feature of the disease because x ray examination has become commonplace During the war primary atypical pneumonia was intensively studied under Army auspices at Fort Bragg where it was shown to be due to a filtrable agent

The symptoms are relatively nondescript, consisting of cough often protracted fever, and a suggestion of pulmonary involvement on auscultation The blood count shows an absence of the leucocytosis one would expect in bacterial pneumonia and the x ray reveals a fairly characteristic form of consolidation largely central in location and frequently migratory The disease occurs at all seasons of the year but is more common at times when the common cold is prevalent Institution outbreaks among student nurses and other students living in dormitories are frequently observed The diagnosis is supported in half the cases by the demonstration that the patient's serum will agglutinate group O human blood cells in the refrigerator but not in the incubator This is the cold agglutinin test and the sera of patients with primary atypical pneumonia will frequently react in dilutions of 1 40 or greater Moreover, the titers may be found to rise during the later stages of the disease The sera of many cases of primary atypical pneumonia will also agglutinate a particular strain of nonhemolytic streptococcus the so called *Streptococcus* MG (the MacGinnicoccus) Agglutinins are present in approximately half the cases, in three fourths of the severe ones Since agglutination of *Streptococcus* MG by human sera is infrequently met in other diseases, the test has considerable significance The value of both these procedures is of course qualified by the fact that we have at present no certain way of diagnosing the cases and the frequency with which these agglutinins are found can never be determined with certainty until a positive diagnosis is possible

Eaton has isolated a virus from cases of atypical pneumonia that is pathogenic for chick embryos, hamsters, and cotton rats It is distinct from the viruses of influenza and psittacosis Unfortunately only roughly half of atypical pneumonia patients develop antibodies to Eaton's agent and the evidence as a whole suggests that it is not a common cause of primary atypical pneumonia Similar successes have been reported by others but none has been readily confirmed or fully met the standards of proof necessary to establish them as the etiologic agent of the disease

The disease has never been transmitted to human volunteers, which has prompted the suggestion that it has a complex etiology and that a second agent or predisposing factors are necessary as well as the primary agent

Equally mysterious has been the coming and going of atypical pneumonia which first attracted attention twenty years ago, was for a time a rather common cause of epidemics in closed communities, barracks, and residence schools, and has during the past several years been seldom seen The more we know of the variety and ecology of the virus infections the more fascinating the subject becomes and the more fluid the problems

V

VIRUS ENCEPHALITIS AND MENINGITIS

During the early 1930's a number of viruses were discovered in the States that were causing encephalitis The diseases themselves were as "new" as the viruses Previously, epidemic encephalitis had been synonymous with sleeping sickness or von Economo's encephalitis which swept the earth following the First World War Somnolence, oculomotor signs, fever, and meningitis had been the features of von Economo's encephalitis which had left many of its young victims disordered in mind and body The primacy of von Economo's encephalitis is fixed in the Japanese terminology for when the newer forms of encephalitis struck in Japan they were called encephalitis "B" to distinguish them from the original von Economo or "A" form

We learned about the newer kinds of encephalitis in the States by indirection In 1930, Karl Meyer isolated a virus from horses in the San Joaquin Valley, which had died of subacute encephalitis Three years later a similar virus was recovered under similar circumstances in New Jersey The two were compared and found to be antigenically different and therefore were called "western" and "eastern" equine encephalomyelitis viruses (W E E and E E E) Attention was first focused on the equine disease, periodically of great economic importance Thousands of horses have been killed by these viruses in North America

There were early clues to infection among horse handlers but not until 1938 was a human epidemic recognized—in Massachusetts where, during a summer of excessive rainfall, of floods, and many mosquitoes, equine encephalitis occurred in epidemic proportions and, at the same time, severe cases of encephalitis in man Horses and men were equally affected and in the same regions, but few of the patients had been in contact with horses Thus the evidence immediately suggested the identity of the two diseases and a common source of infection other than direct contact Virus was isolated from the nervous tissues of nine fatal human cases and infection proven in others by serologic tests The virus was the eastern variety

The human illnesses had begun very suddenly (in contrast to von

Economo's encephalitis) with high fever, excitement or drowsiness, and convulsions Stiffness of the neck was constant A polymorphonuclear leucocytosis was common and the cerebrospinal fluid was remarkable in that most of the cells, of which there were from 200 to 2,000 per cu mm , were polymorphonuclear leucocytes This is the pattern found in bacterial meningitis but, in the Massachusetts cases, the fluids were sterile Some of the patients became comatose and died within a few days Many of the survivors had residua of mental deterioration or paralysis While all ages were affected, the majority of the patients were young children

The Massachusetts epidemic proved to be highly instructive It had been extremely well studied and prepared us for the larger and more frequent outbreaks that followed in the north central states where the western virus is common Sporadic cases and small outbreaks have occurred in the far west as well, in the years since, and in some of them infection, if not disease, had been widely prevalent

Epidemic encephalitis also appeared in St Louis in the summer of 1933 Of 1,100 patients, some 200 died Several investigators, Ralph Muckenfuss, Charles Armstrong and Leslie Webster, succeeded in isolating the responsible virus, again a "new" virus Later it was learned by serologic tests that the same virus had infected many inhabitants of the little Mississippi Valley village of Paris, Illinois, the previous summer and retrospective diagnoses were made of other outbreaks by means of serologic tests The 'virus of St Louis encephalitis" (S.L.E) proved to be unrelated to the two equine encephalitis viruses Indeed we now know that in its antigenic composition it shares components with the viruses of Japanese B encephalitis, epidemic keratoconjunctivitis louping ill, and others, which as a group stand apart from W E E and E E.E But many members of the former family are epidemiologically and clinically similar to the latter The differences must be borne in mind by the laboratory worker but are not important at the bedside or in the field.

Our human epidemics of acute encephalitis have been seasonal, have occurred during the summer months, during the mosquito season, and we can sketch in our present concept of the diseases by saying that all are transmitted by mosquitoes and that a common source of infection has been wild birds (in Massachusetts in 1938,

possibly pheasants) with mammals, including horses and man less frequent sources of infection The interepidemic survival of the viruses of equine encephalitis and St Louis encephalitis appears to depend on mites which commonly feed on domestic and wild birds and at least one of which has been proved to transmit infection transovarianally Birds are intermediate hosts rather than reservoirs of infection The potential importance of Aedes mosquitoes had been demonstrated in the very beginning (1932) by Kelser who had transmitted equine encephalitis to guinea pigs and a horse by allow ing infected mosquitoes to feed on them *Culex tarsalis* is chiefly responsible for human infections The range of infected birds seems to be broad, new species continue to be incriminated The epidemics are largely hidden, only the severe illnesses attract attention Serologic surveys show many more infections than illnesses If the brain is involved, the illnesses, whatever the virus, resemble the Massa chusetts cases and headache, fever, nausea, and vomiting appear suddenly, and the tremors, speech difficulties, stiffness of the neck and mental confusion follow The Kernig and Brudzinski signs are present Excitement, perhaps delirium or drowsiness, may occur Vertigo was a common symptom of the St Louis cases and spastic paralysis was not infrequent

Many more patients have only mild cerebral symptoms, head ache, stiff neck, and nausea But they may nevertheless have ab normal numbers of cells, in the milder cases predominantly mono nuclear, in their cerebrospinal fluid In the absence of encephalitic signs and symptoms such illnesses are indistinguishable from a dozen other nonbacterial infections of the surfaces of the brain—they are cases of ' aseptic meningitis "

' Aseptic meningitis" is the common denominator of many infec tions and one of the commonest problems referred to the diagnostic virus laboratory Many soon declare themselves to be cases of tu berculous meningitis, a few prove to be due to leptospiral infection The majority are apparently a consequence of virus infection Mumps virus is a relatively common cause, the Coxsackie and poliomyelitis viruses account for many Lymphocytic choriomeningitis virus *char acteristically* causes aseptic meningitis

Lymphocytic choriomeningitis virus was also identified for the first time in the 1930's During his investigation of the epidemic

of St. Louis encephalitis, Armstrong recognized a second "new" virus in one of his monkeys. Its association with human illness was then uncertain but not long afterward the same virus was recovered by Rivers and Scott from the cerebrospinal fluid of two cases of rather severe aseptic meningitis. One was suspected for a time to have a brain tumor. Rivers confirmed the etiologic role of the virus by demonstrating the appearance of neutralizing antibodies in the sera of both patients during recovery. The name of the disease and virus was chosen because the lesion in experimental animals is a round cell infiltrate of the meninges and choroid plexus. For a time the disease was called *benign* lymphocytic choriomeningitis but this qualification was dropped following the identification of fulminating, fatal cases which occur on occasion.

Rivers promptly explored the possibility that lymphocytic choriomeningitis virus was the usual cause of aseptic meningitis and learned that it accounted for only some. He discovered clinical differences between lymphocytic choriomeningitis virus infection and aseptic meningitis as defined by Wallgren. Many of his patients had symptoms of meningitis following influenzal respiratory infections. Coma and convulsions were unusual. Moreover, the lymphocytic choriomeningitis cases characteristically had high cerebrospinal fluid cell counts, more than 1,000 monocytes per cubic millimeter being common. In addition, the patients were chiefly older children or adults and most of the cases occurred during the winter and spring months. In other words, once the disease was defined etiologically, it was possible to better define the clinical manifestations.

The year after Armstrong's isolation of choriomeningitis virus, Traub had detected what proved to be the same agent in a laboratory mouse colony where it existed as a latent infection in biologic harmony with its host. We now know that this may occur in house mice as well. House mice are indeed the usual source of human infection. Families or colonies of mice have remained infected through many generations. We ourselves have repeatedly identified human cases with particular buildings in which the mice were infected. In several instances human infection recurred in the same buildings for many years.

The practical point is that lymphocytic choriomeningitis, as other forms of aseptic meningitis, has clinical and epidemiologic charac-

teristics that are helpful in diagnosis In the Albany laboratory we sometimes suspect lymphocytic choriomeningitis from the patient's address for we now know which districts are infected If the address and history are suggestive, we begin by inoculating mice with cerebrospinal fluid, the method of choice in the diagnosis of choriomeningitis There are few clues to mumps meningitis other than the prevalence of mumps parotitis Coxsackie virus infection, like poliomyelitis, is seasonal and may be recognized clinically with certainty if the pathognomonic mouth lesions of herpangina or typical symptoms and signs of Bornholm disease are associated Mosquito-borne encephalitis of North America is restricted to certain geographic areas, the central and far western states being the major foci of infection Human infection has never been identified in New York and has been infrequent in most of New England Moreover the disease itself, at least when seen in an epidemic, is distinctive In other words, laboratory diagnosis is more efficient and productive if advantage is taken of clinical and epidemiologic information in planning the examination

Unfortunately these methods of arriving at a good guess are limited by the tendency of many of the infections to occur simultaneously and under similar circumstances Minnesota and the Dakotas have summer epidemics consisting of both equine and St Louis encephalitis, frequently with poliomyelitis as well The Coxsackie and poliomyelitis viruses go hand in hand as a rule, their epidemics are usually mixed Physicians need to be as critical of the epidemiologic as the clinical evidence Indeed this may be a situation where a more intensive circumscribed epidemiologic and clinical examination might be expected to have advantages Meanwhile, etiologic diagnosis, as arrived at in the laboratory, should provide facts by which clinical impressions may be tested And it is quite possible that the more general application of laboratory tests, especially the isolation of the agents, will disclose other still unrecognized viruses capable of inducing similar illnesses

The viruses of equine encephalomyelitis and lymphocytic choriomeningitis are small, approximately 40 mμ, that of St Louis encephalitis is still smaller, 25 mμ All are readily isolated from the central nervous tissues of fatal cases, from the blood during the onset of illness and, in the case of lymphocytic choriomeningitis in particular,

from the cerebrospinal fluid for a number of days The sera of convalescents fix complement in the presence of virus antigens which are now commercially available and all induce the formation of neutralizing antibodies which may be demonstrated in simple animal experiments All have a broad host range This is especially true of the "equine" viruses Suckling mice are exquisitely susceptible to all but lymphocytic choriomeningitis virus In its isolation, weaned mice must be used, for the others, younger animals are better Whether this unusual reversed age susceptibility has some association with the innocence of natural choriomeningitis infection in mice we do not know Suckling mice are fully susceptible to infection once the freshly isolated strain has been passaged several times Adult mice are uniformly susceptible and behave in a highly characteristic, pathognomonic manner before death A presumptive diagnosis may be made from the appearance of the test animals

Man is not the natural host but only an accidental victim of little importance in the natural history of the viruses In the natural host, infection is a benign, slumbering affair There are other viruses that fortunately do not occur in the States, which closely resemble these agents Japanese B encephalitis, for example, is a common disease in eastern Asia, as well as Japan and is known in Australia and the Philippines Horses and pigs are frequently infected as well as man The Russians have a similar disease, Russian spring summer encephalitis, the agent of which is transmitted by ticks Ticks are the vector, too, of louping ill, a disease of sheep that is at times transmitted to man All of these viruses share common properties with the virus of St Louis encephalitis Rather similar viruses have been isolated in Africa and tropical America, usually from mosquitoes They appear less pathogenic for man although few of them have as yet been fully assessed

The natural history of the arthropod borne diseases approximates that of yellow fever which also has its jungle reservoir but is carried at times to man by blood sucking insects It has been suggested that poliomyelitis (and presumably the Coxsackie viruses as well) may have arisen in the jungles but their present characteristics deny it For them, man himself appeared to be the reservoir both are well adapted to the human host

Certainly there is little justification for considering the encephali

TABLE 1

PRINCIPAL DISEASES IN WHICH VIRUSES HAVE BEEN ISOLATED DIRECTLY FROM THE HUMAN CNS

Virus	*Epidemiological Character*		*Probable Source of Human Infection*			*Most Characteristic Type of Illness*		
	Predominantly Epidemic and Seasonal	*Predominantly Sporadic, Non-seasonal*	*Human Contact*	*Biting Arthropods*	*Secretions or Excretions of Animals*	*Encephalitis or Encephalomyelitis*	*Myelitis*	*Aseptic Meningitis*
Poliomyelitis	x		x				x	
St Louis encephalitis	x			x		x		
Western equine encephalitis	x			x		x		
Eastern equine encephalitis	x			x		x		
Venezuelan equine encephalitis	x			x		x		
Japanese (B) encephalitis	x			x		x		
Russian spring-summer enc	x			x		x		
Louping ill	x			x		x		
Lymphogranuloma venereum		x	x			x		
Herpes simplex		x	x			x		
Mumps		x	x					x
Rabies		x			x	x		
"B" virus		x			x	x		
Lymphocytic choriomeningitis		x			x			x

Schlesinger, R W In *Medical Monographs*, Series I W W Bean ed Baltimore, Williams and Wilkins 1952 p 382

tides as "new" in any sense other than that they were new to us Australia experienced a rather large and deadly epidemic of encepha litis during the First World War and Japan was ravaged by an epi demic in 1924 Both of these outbreaks can be identified as typical examples of arthropod borne encephalitis with considerable assurance The agent responsible for the Australian disease (Australian X disease) was transmitted to monkeys, sheep, and horses but subse quently lost Louping ill virus, also capable of causing encephalitis in man, has been well known as an enzootic of sheep for more than 100 years

VI

THE POLIOMYELITIS VIRUSES AND THEIR COUSINS

ONE CANNOT do justice to all that has been learned about polio myelitis in a single chapter And yet we do not know enough to control it This has often happened in the past When we find the solution we can, perhaps, disregard many of the routes that are now being explored and forget the endless experiments that con tributed to the solution

The poliomyelitis problem has been primarily a question of patho genesis Infantile paralysis was first considered to be a rare, *essential* form of paralysis of infancy Once the characteristic lesion was rec ognized it became evident that the disease was infectious The nature of the etiologic agent was proven by Landsteiner who reproduced the lesion in monkeys by inoculating them with an aqueous suspension of spinal cord of a fatal human case For years the rhesus monkey was the experimental animal of choice and the impression was gained that the virus was strictly neurotropic This seemed to imply that exposed olfactory nerve fibers were the portal of entry for only the olfactory nerves are open to the environment There were contrary observations at the time, the successful isolation of virus from mesenteric lymph nodes and Kling's experiments that pointed to an intestinal phase of infection but they were generally disregarded until Trask demonstrated that virus may often be found in the feces of patients and carriers The olfactory route of infection was further discredited when it was learned that, while chemicals sprayed into the nostrils of monkeys temporarily protected them from virus instilled intranasally, children were not so protected Pathologists might have foretold this for the olfactory nerves are rarely diseased in human poliomyelitis

The final capitulation of the neurotropists followed Enders' suc cessful growth of the virus in tissue cultures of skin, muscle, and connective tissue cells and proof that viremia precedes involvement of the central nervous system in cynomolgous monkeys Viremia has also been demonstrated in human poliomyelitis but only infre quently The difference may owe a great deal to the practical diffi

culty of investigating human infections The implications of these two discoveries have been very important for they indicate that poliomyelitis is not unlike many other virus infections with an early stage of multiplication followed by viremia and localization in susceptible tissues This would mean that the virus is not protected by an intranervous system location from the start and that humoral immunity might block infection of the nerve cells and thus prevent the paralytic complications of poliomyelitis virus infection

It would be both unfair and incorrect to deprecate the gain made by the study of poliomyelitis in monkeys Neutralizing antibodies were demonstrated and the prophylactic value of immune serum was proven Indeed immune serum given intrathecally soon after infection was shown to abort experimental poliomyelitis and this led to extensive human therapeutic trials of immune sera which largely failed Vaccines too were early developed using infected monkey cord as antigen These also failed in the field—whether because of their feebleness or because they were antigenically too narrow we do not know

Armstrong's adaptation of poliomyelitis virus to the mouse shook off some of the shackles and made possible extensive measurements of the humoral immunity of large population groups and inevitably drew attention to the differences between poliomyelitis viruses Later it was learned that there are at least three distinct types the Brunhilde (Type 1) the Lansing (Type 2) and Leon (Type 3) which in the States occur in that order of frequency There may well be others for strains nonpathogenic for monkeys may exist and may some day be isolated by means of tissue culture or some animal other than monkeys Not all cases of poliomyelitis can as yet be ascribed to infection with one of the three known types

We now believe poliomyelitis to be a common enteric infection that is transmitted to contacts in most cases as fecal virus, at times by nasal secretions It is an infection limited to man, one that readily immunizes infants at small risk of clinical disease but that in older susceptibles not infrequently travels via the blood stream or along nerve fibers to invade and selectively destroy motor neurons and thus cause flaccid paralyses We now know too thanks to Hammon that single doses of immune globulin prepared from human serum pools are capable of preventing clinical poliomyelitis in many ex

posed individuals for as long as six weeks and we infer that a relatively low level of acquired humoral immunity should also protect The insusceptibility of adults may be due to acquired immunity which would confirm the theory It is anticipated that vaccines capable of providing a similar level of antibody would be equally effective for longer periods But only the future will tell whether vaccines of killed virus will stimulate sufficient response or whether a living but modified virus is needed Virologists, remembering yellow fever, are inclined to pin their hopes on a living vaccine and trust that such a one will soon be found Furthermore, there are reasons for hoping that an immunization capable of preventing intestinal infection which, judged by monkey experiments seems rather dissociated from humoral antibody, may be developed for it would provide better prospects of controlling infection and reducing the number of excretors

Poliomyelitis usually begins as a rather nondescript, minor illness consisting of fever, sore throat, or mild gastrointestinal symptoms and headache These complaints disappear for a day or more to be followed by more severe headache, stiff neck and back, and frequently pain in certain muscles At times the latter symptoms develop abruptly without prodromes or close on their heels A clinical diagnosis at this stage is at best presumptive, supported perhaps by the coexistence of paralytic poliomyelitis in the community Laboratory evidence of infection by poliomyelitis viruses has at times been secured from such cases and similar illnesses have been experimentally produced in chimpanzees but the isolation of virus can hardly be considered certain proof since other infections, notably by the Coxsackie viruses, may account for the symptoms

Increasing muscle tenderness, perhaps with weakness, and a cerebrospinal fluid pleocytosis more strongly suggest poliomyelitis and the occurrence of flaccid paralysis presumably proves it The cells in the cerebrospinal fluid are, in the earliest stages, usually polymorphonuclear in type, but with the onset of paralysis the more typical finding of mononuclear cells is the rule These seldom exceed 500 per cu mm and occasionally an increase of cells is not noted at all The protein is increased moderately but the spinal fluid sugar is unchanged

At this time clinical diagnosis may be certain especially when

viewed against the circumstances, the season of the year and the absence of a typical sign of another disease In truth, therefore, much depends on the recognition of paralysis and the examiner should train himself to be thorough and careful in seeking this important diagnostic sign Especially, evidence of paralysis of the diaphragm and abdominal muscles should be sought since paralysis here is more difficult to recognize and yet its presence is extremely important not only because these are common sites of paralysis but, occurring in the earlier stages, often warn of extensive paralysis of the extremities yet to come Comparison of the two sides is helpful, as well as viewing the motions of the abdomen and thorax under controlled light The frequency of paralysis varies greatly in different epidemics but it also varies greatly with examiners The individual muscle groups of the extremities and head should be separately tested, respiration and swallowing carefully observed to detect the beginning of bulbar paralysis

Of all the diseases that may resemble poliomyelitis, radiculoneuritis or the Guillain Barre syndrome is most likely to cause uncertainty The most helpful differential sign is the presence of increased cerebrospinal fluid protein without an increase in cells but the absence of prodromes and the more gradual yet progressive course are also uncommon in poliomyelitis Paralyses are less complete in radiculoneuritis and more likely to be symmetric Pain is less severe and paraesthesia is more common Involvement of the urinary bladder has in our experience been much more common in radiculoneuritis than in poliomyelitis

Localized muscle tenderness and pain occur in Bornholm disease but presumably never flaccid paralysis Nor is the pain as protracted Poliomyelitis is often a most painful disease and the hurt and spasm in affected muscles may be excruciating This is a symptom that seems to respond best to hot packs, and the comfort of heat and relief of spastic splinting seem to be the explanation of Sister Kenny's successes in the treatment of poliomyelitis

The outcome of poliomyelitis is related to the age of the patient, complete recovery being nearly twice as common in infants as young adults and deaths but one-fourth as frequent In individuals this tendency needs to be evaluated in terms of the extent and course of the paralysis and especially the presence of bulbar paralysis which

is the usual mechanism of death The outcome varies greatly from year to year, certain epidemics having been marked by mortality rates as great as 40 per cent and others by very few deaths or permanently crippled patients Whether the strain of virus is chiefly responsible for these variations we do not know

As to treatment, it is clear that lives may be saved by highly expert care of the patient with respiratory paralysis, the anesthesiologist is the man most needed The Kenny treatment provides comfort in the acute stage, and sensible care, exercise, and encouragement sometimes work wonders in restoring the crippled

The typical lesion of poliomyelitis is an acute degeneration or death of motor neurons of the anterior horn of the spinal cord, associated with inflammatory reactions about the vessels and interstitium These lesions are usually diagnostic If similar changes occur in the motor areas of the cerebral cortex, in the medulla and pons, the cerebellar hemispheres being spared, the diagnosis may be considered to be above reproach Interstitial myocarditis occurs in perhaps 15 per cent of all fatal cases of poliomyelitis Virus has been recovered from the heart and at times, from the blood It may frequently be found in the medulla and spinal cord Enlargement of the lymphatic tissues of the intestinal tract is frequent and in fatal cases aspiration pneumonia and rupture of alveoli bear testimony to the manner of death

We have no present prospects of effectively treating poliomyelitis Successful therapy would seem to be the practical answer if it might be found We can confer passive immunity by means of immune globulin but the difficulties of applying control by such means are tremendous Active immunization seems the most promising next step A more desirable solution would be the control, the suppression of infection Poliomyelitis is an enteric disease and our enteric disease vaccines have been notably less effective than many others The protection afforded by active immunization might well be precarious if infection persisted to high degree for every defect in immunity would allow infection to break through These are problems that cannot be solved short of prolonged observation in the field

A connected problem has been created by the discovery that there are other viruses rather similar to the poliomyelitis viruses These should be briefly considered both because of their theoretical and practical significance

The Coxsackie viruses

A large family of viruses, the Coxsackie viruses, are much like the poliomyelitis viruses While we know of but three poliomyelitis viruses we have thus far discovered 24 Coxsackie viruses Poliomyelitis and Coxsackie viruses are both small, probably 30 mμ in diameter, occur most often and longest in the feces, are common human infections, have no other known host Both may often be found in sewage in late summer Both occur, but briefly, in the throat Both are resistant to changes in pH to phenol and survive for days at room temperature and indefinitely in the frozen state Their inactivation by heat (53° 55°C) is comparable to that of poliomyelitis virus

Infection by both is most prevalent during the later summer months, chiefly among the young Indeed, so far as we know the epidemiology of the two is identical Both induce neutralizing and complement fixing antibodies but in both these are of little value in diagnosis because they occur irregularly in time and degree among the sick and the well

The Coxsackie viruses have complicated poliomyelitis by forcing us to re-examine the evidence we have regarding cause and effect It seems most probable that crippling or fatal poliomyelitis is due to infection with poliomyelitis virus but it is by no means clear whether the same is true of the clinical entities, abortive and non paralytic poliomyelitis

Medicine progresses by further definition of disease and more precise classification In the days of Victoria and Albert, William Jenner distinguished between typhoid and typhus fever, between slow nervous, and putrid fever This was not an easy distinction on clinical ground when the two were occurring simultaneously but now that we know of their etiology and pathology the differences are striking Since then typhus fever itself has been subdivided into murine and Old World typhus, Rocky Mountain spotted fever, scrub typhus, and rickettsialpox Something similar seems to be occurring in the case of poliomyelitis

The Coxsackie viruses force us to re examine the virus infections of late summer, of the poliomyelitis season A few years ago these were generally considered to be predominantly due to poliomyelitis virus We now know that other viruses are involved and that the epidemics are frequently mixed

The problem requires close study For example, late summer epidemics have been investigated (and we exclude from the present discussion epidemics of mosquito borne encephalomyelitis and other well known entities) that have the features of extremely mild poliomyelitis Rather characteristically under such circumstances poliomyelitis virus is either not isolated or has been but rarely isolated Coxsackie viruses have frequently been recovered although not regularly enough to suggest they have been responsible for the epidemics In some instances double infections have been proven To add to our confusion there remains the possibility that other, still unrecognized viruses may have played a part Indeed, such are now being found in significant numbers, thanks to tissue culture technics

Illnesses resembling abortive poliomyelitis not uncommonly are intimately associated with Coxsackie virus infection and sometimes occur as small outbreaks free of any suggestion of paralysis Intensive study is required to resolve these cases and epidemics and to exclude poliomyelitis

This then, is the problem of the Coxsackie viruses as they affect our understanding of poliomyelitis They have other roles as well Two groups of Coxsackie viruses have been recognized The Group-A varieties, of which 19 types are known, are easily recognized because they cause widespread degeneration of the skeletal muscles of infant mice The Group B viruses cause not only focal muscle lesions but encephalomalacia, and a unique inflammation of fat pads as well At certain ages necrosis of the acini of the pancreas may also be found Both varieties are fully pathogenic only for immature mice, the favorite experimental animal, although pancreatic necrosis can be induced in adult mice by Group-B strains This tropism is intensified by carcass suspension transfers

Group A Coxsackie viruses are responsible for herpangina, a disease chiefly of young children during the last months of summer characterized by multiple herpetiform blisters of the soft palate and posterior pharyngeal wall, fever, headaches, and at times pains in the muscles The blisters are small, 2 to 3 mm in diameter, surrounded by a thin, bright red rim of inflammation They quickly rupture They do not occur on the lips or gum as do herpetic sores and are more evanescent Herpangina was proven to be due to infection by several types of Group A Coxsackie virus by Huebner and his as-

sociates who established that infection was common in patients and absent from healthy children of similar ages In other words, they emphasized the significance of their virus isolations by means of suitable controls Moreover, the mouth lesion was shown to be highly infectious

Group-B Coxsackie viruses cause epidemic pleurodynia or Bornholm disease, also a disease of later summer, characterized by the sudden onset of excruciating pain, most commonly in the lower thorax but often in the abdomen or an extremity or the shoulders Bornholm disease and herpangina are usually accompanied by severe headache and frequently cases of the former at least have symptoms of aseptic meningitis, the cerebrospinal fluid cells are increased in number Whether these are cases complicated by poliomyelitis virus infection or whether the Coxsackie viruses have invaded the central nervous system has not been proven

Bornholm disease, apparently in distinction to herpangina, has a tendency to relapse, and recurrent cases and chronic forms have been described including a number of remarkable instances in which muscle biopsy yielded not only virus but lesions similar to those in mice A swollen, tender muscle may at times be found in the early stages of Bornholm disease, further evidence of the similarity between it and experimental Coxsackie virus infection

The association of Coxsackie viruses with Bornholm disease was first perceived by Curnen while investigating an epidemic of Group B 1 Coxsackie virus infection It is interesting to note that very few of the illnesses resembled Bornholm disease, the majority were cases of "nonparalytic poliomyelitis" The responsible type was frequently isolated in New York the same year but none of the illnesses resembled Bornholm disease This type has not since been found in New York During 1950 and 1951, Bornholm disease was prevalent in our western states and in Great Britain and Europe In all of these cases it was associated with a B 3 type of virus It would seem that the epidemiology of the Group-B Coxsackie viruses may be unusually interesting and instructive, the outbreaks being widespread but infrequent In contrast, several of the Group-A strains have recurred year after year or at two- or three-year intervals

The Coxsackie viruses may be responsible for other diseases They appear to have been the cause of an epidemic of encephalomyelitis

in Australia in January, 1951, and have been associated sporadically with radiculoneuritis

Coxsackie virus infection is unique in that it is most easily diagnosed by isolation of the virus The serologic tests are cumbersome because of the number of types of viruses that must be considered It is likely that once the responsible strain has been isolated serologic tests would prove useful in surveying other cases A not infrequent difficulty in isolation occurs if both a Group A and a Group-B virus are present In that case the Group A component must be neutralized with type specific antiserum to afford the B an opportunity to develop, to be recognized, and isolated

The EMC viruses

Intricately involved in the poliomyelitis literature are a number of viruses frequently called the EMC or encephalomyocarditis viruses They form a bizarre story The first to be discovered Columbia SK virus, was established in mice by Jungeblut and Sanders who undertook to repeat Armstrong s adaptation of Lansing poliomyelitis to mice The virus proved to be different in a number of respects from Armstrong's virus Its infectivity titer was much higher and it had largely lost the faculty of causing paralysis in rhesus monkeys Moreover it was not definitely neutralized by pooled adult human sera as poliomyelitis viruses are Many experts considered it was a mouse virus or pickup from the mice or cotton rats used in the adaptation

A few years later the same agent or a very similar one was isolated from a fatal case of poliomyelitis This strain (MM) was not neutralized by convalescent sera from the epidemic nor by random human sera from New York and its origin was therefore unclear Still later a similar virus (Mengo) was isolated in Africa from mosquitoes and from a physician ill with encephalitis It has also been recovered from chimpanzees that had died of encephalitis and acute myocarditis in Florida (EMC) The agent is believed to have been the cause of an epidemic of "nonparalytic poliomyelitis" in the Philippines A number of similar viruses have been isolated in Western Germany and the Netherlands Some of these differ somewhat in host range from the original strain but all are antigenically alike

Jungeblut has from the first considered these viruses to belong to

a family of poliomyelitis viruses and distinguished them as "murine" poliomyelitis viruses They are capable of causing flaccid paralysis and anterior horn neuron degeneration in cynomolgous monkeys, to a lesser degree in rhesus monkeys They are antigenically different from the known poliomyelitis viruses Thanks to Hallauer, we know that they hemagglutinate sheep red blood cells The poliomyelitis and Coxsackie viruses do not do this

It was proposed by Mollaret that they be included in the family of poliomyelitis viruses as a group of para poliomyelitis viruses This suggestion has won only limited support possibly because there is as yet inconclusive evidence that they are connected with human poliomyelitis and in fear that such a term would confuse the issue rather than clarify it Meanwhile it has become clear that these viruses are not uncommon infections of man Specific antibodies have been found in a fair proportion of individuals in Western Germany who had recently suffered from central nervous system diseases of uncertain nature (but not poliomyelitis) Antibodies have been found in random sera collected in Mexico It is noteworthy that convalescent poliomyelitis sera collected in the same area rarely contain such antibodies while rat sera both in our southern states and in Mexico frequently contain antibody

The EMC viruses do not seem to be causally related to paralytic pol[illegible]

[illegible]

ment serologic tests are simple and reliable and isolation might be expected to be straightforward They resemble the poliomyelitis and Coxsackie viruses in size and in resistance to physical agents but have only once been demonstrated in human feces They should perhaps be considered whenever the circumstances point to a close association of patients with aseptic meningitis and rats

VII

VIRUS INFECTIONS OF SKIN AND BRAIN

THREE common viruses of fairly large size characteristically cause vesicles of the skin and mucosa and parasitize neurons Two are related The third and somewhat smaller one is quite distinct but may be properly associated with them by the nature of the lesions it induces Although all three have been known for many years as human infections we still know relatively little about them and their further study in hospital laboratories may be very instructive

Herpes simplex

The virus of herpes simplex is the smaller and simpler to isolate It is readily recoverable from the common cold sores that plague so many people, the blisters of herpes fibrilis, and from cases of herpetic stomatitis as commonly seen in infants In the latter at least it is often simpler to isolate virus from the feces than from the vesicles within the mouth for these ulcerate so quickly that it is difficult to collect suitable specimens Fecal herpes simplex virus seems to be excreted virus, not virus that has multiplied in the intestinal tract as poliomyelitis virus is known to do Obviously the virus is resistant to intestinal enzymes but it is doubtful that it is an enteric infection Isolation is readily accomplished by inoculating suckling mice (intracerebrally) or the chorioallantoic membranes of fertile hens' eggs In baby mice the virus causes an acute, fatal encephalitis within the week, which is easily identified histologically since the typical intranuclear inclusion bodies occur in profusion On the chorioallantoic membrane (CAM), the virus produces small pocks and later infection and death of the embryos Adaptation to suckling mice is easier than to the egg

Stomatitis in the young may be an acute febrile disease with extensive ulcerations of the gums and mouth The cheeks are most severely involved and the blisters quickly ulcerate and are replaced by a dirty membrane In these extensive and severe infections virus can be easily demonstrated in the oral secretions as well as the feces

Herpes simplex virus was originally demonstrated by infecting the cornea of rabbits In this case the superficial cells carry inclusion

bodies and the infection causes a keratitis Herpes zoster virus is not pathogenic for the rabbit cornea and the two may be distinguished in this way Rabbits may also be infected intracerebrally but domestic rabbits sometimes carry herpes virus as a latent infection In such animals even sterile inocula can precipitate encephalitis which makes the intracerebral test somewhat unreliable

Herpes virus is capable of causing a severe encephalitis in youngsters In these cases isolation and identification of the nature of the infection have usually been made by inoculating animals with brain tissue suspensions The inclusion bodies in the fatal human cases may be infrequent and overlooked Presumably milder forms of infection also occur in the brain and herpes simplex infection must be considered as a possible but uncommon cause of illness ranging from aseptic meningitis to fulminating and fatal encephalitis Efforts should be made to recover virus from the cerebrospinal fluid and to compare the antibody content of sera collected early and late Virus neutralization tests (using mice or eggs) and complement fixation are recommended for serologic tests and for the antigenic identification of herpetic viruses Tissue culture technics promise great usefulness although few random isolations of herpes simplex virus have turned up in the extensive testing that has been made for Coxsackie and poliomyelitis viruses

One probable explanation of this is the character of herpetic infection which is predominantly latent Indeed herpes virus is the most striking known example of latent virus infection The evidence we have points to life long carriage in some individuals This accounts for the common story of repeated bouts of fever blisters and persistent and recurrent herpetic keratitis in infected individuals whenever precipitating factors appear, and the persistence of serum antibodies Whatever the haven of the virus may be, it most effectively protects it from humoral antibodies because virus exposed to antibodies is promptly neutralized

Herpes zoster

The vesicles of herpes simplex *occasionally* are associated with nerve distribution those of herpes zoster which is caused by a quite different virus are *regularly* associated Moreover zoster is a very painful affliction because the lesion typically involves nerve ganglia with spread of virus to the skin via nerve fibers Both

watery vesicles to form in the superficial corium The zoster vesicles not only are associated with the distribution of sensory nerves but are in almost all cases unilateral, a feature of great value in judging the nature of atypical lesions (papular or simply erythematous) Herpes zoster is a febrile disease and if upper nerve ganglia are involved paralysis is frequently seen as in the larynx While "shingles" commonly occurs along intercostal nerves, the painful, unilateral involvement of many other nerves with vesiculation is strongly suggestive of herpetic infection The perineum and vagina the pharynx and tongue are susceptible to herpes zoster virus infection Significantly, herpes virus has at times been isolated from the cerebrospinal fluid of patients with shingles

Varicella

Chickenpox is caused by a virus related to that of herpes zoster but, whereas chickenpox is a very common experience of young children and is uncommon in adults (with the exception of those raised in isolated areas), herpes zoster characteristically occurs in adults However, both have been seen simultaneously in children and indeed the association of the two in closed groups and the occurrence of varicella in children in contact with adults suffering from zoster is testimony to the fact that both viruses are capable of causing both diseases Evidence of this quality has been repeatedly reported and promises that antigenic relationships will some day be demonstrated under controlled conditions

Chickenpox is of course, a common epidemic disease with a well established incubation period (usually two weeks), a brief prodromal interval followed by crops of papules and vesicles on the face and trunk and lymphadenopathy The local lesions are indistinguishable from those of herpes zoster although they lack the characteristic distribution Varicella is a generalized infection and typical lesions occur internally as well as on the skin and mucosa Its generalized nature and pleomorphic skin lesions resemble smallpox and the two were *not* separated until the last century Chickenpox has a continuing importance as a possible source of error in diagnosis since atypical smallpox may easily be confused with it A good rule to bear in mind is that smallpox should at least be considered in all cases of chickenpox occurring after the fifteenth year of age Under suspicious cir

cumstances, as when the patient believes he once had chickenpox, a laboratory search should be undertaken for variola virus

Biopsy is used surprisingly little in the study of the poxes and exanthemata although the histologic structure of the lesions is usually revealing and diagnostic Microscopic examination will not, however distinguish between varicella and herpes zoster and the intranuclear inclusions (Tyzzer inclusions in varicella and Lipschutz bodies in zoster) are indistinguishable as is the nature of the vesicle and the degeneration of the prickle cells which form it

Two other interesting and important virus diseases indicate their presence by skin lesions in these cases by infiltrations rather than by vesicles They are measles and German measles or rubeola and rubella It is quite likely that an additional form of measles occurs Observations in the field suggest the existence of at least one additional type

Measles

The history of measles is a fascinating story The initial experimental work started in 1758 was influenced by the successful practice of variolation Francis Home a Scotch doctor took blood from a superficial vein of measles cases soaked cotton threads with it, and bandaged the threads to the abraded or scratched skin of fifteen volunteers Seven of these developed typical measles In 1905 Ludwig Hektoen inoculated two volunteers who likewise developed measles 11 to 13 days later It has since been shown that monkeys are susceptible to measles virus but since monkeys are relatively impractical laboratory animals and no better experimental host has been found in which the disease may be reproduced the study of measles has progressed slowly

That measles is caused by a virus is certain The agent passes filters that restrain bacteria and has been grown in tissue culture and in eggs Unfortunately the chorioallantoic membrane does not produce a typical lesion but propagation has been demonstrated So far the experience has been that virus grown in either fashion loses its pathogenicity for men and monkeys and after a few passages in eggs or tissue culture is incapable of producing typical measles In some instances a modified milder form of the
occurred and it was hoped that a method of this kind

a satisfactory vaccine Unfortunately, the mild, modified form of measles does not produce a solid immunity This has been a major obstacle to the use of an attenuated virus vaccine

The natural history of measles is also fascinating Measles is an extremely common infection It is likely that at least 85 per cent of adults in North America have had measles and become immune to it The epidemics which occur in the lower grades of our schools typically recur every third year, sometimes biennially The implication is that following an epidemic the virus dies out because too few susceptibles remain in the community to perpetuate the epidemic In the interepidemic periods the virus survives by the infection of an occasional nonimmune and this continues until the population of susceptible children in a school becomes sufficiently large to support a fresh epidemic It is the replacement of susceptibles that determines the biennial or triennial periodicity of measles epidemics

Clinical measles illustrates a striking age effect, something that is referred to elsewhere in terms of experimental virus infections It is not only that younger children are more susceptible but that the disease in the very young is more severe Indeed measles in the very young is a serious affair and children under three years of age should be protected by passive immunization (gamma globulin) This completely protects half and reduces the severity of the disease in many others

The clinical signs of measles begin with sneezing, coryza, and conjunctivitis often with photophobia, of several days' duration followed by the appearance of specific whitish lesions (Koplik spots) opposite the first molars in the mouth These are blue white, pinpoint lesions surrounded by red areas and their occurrence is followed by the appearance of a diffuse rash over much of the skin surfaces beginning as a rule on the face and neck and proceeding downward over the trunk Uncomplicated measles of this kind is not a severe disease excepting in infants and by the time the rash appears the fever usually vanishes and the patient makes a rather uneventful recovery However, of theoretical as well as practical importance, a considerable number of patients subsequently develop pneumonia and perhaps one in a thousand, encephalitis Evidently measles, as so many of the viruses that parasitize the skin, has distinct neurotropic tendencies That measles virus also has an affinity for the epithelium of

the respiratory tract is of particular importance in view of the lesions and the pathogenesis of the disease

The histologic pathology of measles is instructive The lesion is specific This is true of the Koplik spots in the mouth as well as similar lesions which may occur in the respiratory epithelium and throughout the gastrointestinal tract Whatever the location, they consist of inflammatory changes in lymphoid tissue characterized by the presence of huge giant cells which may contain as many as 100 nuclei (Warthin Finkeldey cells) These are frequently found in the appendix and an occasional child in the prodromal stages of measles with abdominal pain is explored for evidence of appendicitis and pathologists are therefore occasionally able to 'predict" measles by identifying the specific lesions in an excised appendix, before the appearance of the rash The giant cells represent the fusion of many cells their nuclei are assembled from cells that have coalesced In addition to these monster cells, acidophilic inclusions occur in the cytoplasm of epithelial cells and those lining the bronchi may fuse into long, flat, giant cells that resemble combs In view of this effect in the respiratory epithelium, it is hardly surprising that it becomes susceptible to secondary infection and that formerly pneumonia caused many deaths among cases of measles Of hospitalized patients, fully one-fourth develop pneumonia of some extent but the killing character of measles pneumonia is now controllable by antibiotic treatment

The neurotropism of measles virus is suggested by the occasional occurrence of encephalitis Nothing need be said of the symptoms which are the same as those associated with other neurotropic virus infections Symptoms of encephalitis following measles characteristically appear in the week following the appearance of the rash Recovery is the rule

The control of measles by quarantine is practically impossible because patients are most infectious during the prodromal stages when recognition is uncertain The youngster in the stage of conjunctivitis and sneezing is most dangerous to his playmates but the diagnosis of *measles can seldom be made with certainty at that time* This may account in part for the ease with which measles virus spreads through a community

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tation of the virus to tissue cultures promises improvement A simple and apparently reliable microscopic diagnosis may be made during the prodromal period by aspirating nasal mucus and spreading it on a slide and staining by the well known Papanicolaou technic The giant cells that occur in the appendix and elsewhere may also be found in such preparations Clinical diagnosis is usually straight forward although many experts suspect that other yet unidentified viruses will be found that cause similar diseases

German measles (rubella)

German measles is a milder disease than rubeola and is less contagious Therefore immunization of the young adult population is less complete and this has serious implications

German measles is caused by a virus The disease has many characteristics of virus infections and the agent has been filtered and the infectivity of the filtrates proven by transmission experiments using susceptible human volunteers In addition the disease, in a modified form, has been transmitted to monkeys and cultivated in fertile hens' eggs However, as in measles, no satisfactory experimental animal has been discovered and the laboratory study of measles is extremely limited

The prodromal period of German measles is somewhat shorter than that of rubeola Usually only 24 hours of catarrhal symptoms occur before the appearance of the rash which is quite similar to that of measles except in its distribution The face, neck, mouth, wrist, and chest are first affected The rash spreads rapidly and recedes on the third day The face is usually free from rash by the time the legs are involved The importance of Koplik's observation was that it provided a differentiation on clinical grounds of measles and German measles The lymph nodes below the occiput are enlarged and shotty

The occurrence of encephalitis following German measles (in certain instances the encephalitic symptoms have preceded the rash) is relatively rare but is known to occur *It is a token of the neurotropism of the virus Of greater importance* is the observation of increased susceptibility to German measles of the human embryo In 1941 Gregg, an Australian ophthalmologist, noted that several of his young infant patients with cataracts of their eyes, had been born of mothers who remembered having had German measles during pregnancy Gregg investigated the circumstances in great detail and discovered that women who had had German measles during the

first trimester of their pregnancy frequently gave birth to infants with congenital cataracts, anomalies of their hearts, or who were mute or microcephalic. This significant observation has been confirmed repeatedly In some series of cases 85 per cent of all pregnancies complicated in the first trimester by measles were found to be associated with one or more congenital defects and that 40 per cent of pregnancies that had been complicated in the second trimester had similar sequelae Other studies notably those in Sweden, have placed the incidence at a lower figure The Swedes estimate that half of the infants born of mothers early exposed to German measles have congenital defects The cataract is due to massive necrosis of the nucleus of the lens The cardiac anomalies include patent intraventricular septa and foramen ovale The mutism varies in its severity in part depending on the period of pregnancy in which the disease occurred Mental retardation has frequently been noted and diaphragmatic hernia has been described Abnormalities of the teeth appear to be another common result of maternal rubella and consist of delayed eruptions, imperfect enamel and a predisposition to caries.

The full significance of Gregg s observation may not yet have been measured Various steps have been taken to meet the situation In Sweden and Denmark young mothers who contract German measles during the first months of pregnancy are usually aborted If the exposure is recognized and immune globulin administered promptly it may be possible to prevent defects in that way A further suggestion has been that 18 year old girls be deliberately exposed to German measles before they marry and conceive and the establishment of special homes where young women could be infected has been recommended Since the virus is not satisfactorily cultivated in the laboratory, there are practical difficulties to this plan one of which would be the provision of a supply of German measles virus that was surely free of other viruses Of perhaps greater importance especially in view of the scanty information we have regarding comparable virus infections is the possibility now supported by considerable suggestive evidence, that similar lesions in infants are at times caused by other virus infections during pregnancy Some of these may be less conspicuous than German measles Possibly further development of our knowledge of minor virus infections will provide means for the further prevention of congenital defects

VIII

RABIES

One might very well assume that rabies exists precariously Infected animals invariably die and rabies virus would die with them if meanwhile the animal did not directly and conspicuously infect another by biting it Despite this clumsy means of propagation, or, perhaps because the virus has a safer haven elsewhere that we do not know about (as in bats?), rabies has survived for at least 4,000 years, for as long ago as that the law required "If a dog is mad and the authorities have informed its owner, but he does not cage it, if it then bites a man and causes his death, the owner of the dog shall pay two thirds of a mina (40 shekels of silver)"

From this and other testimony we may conclude the disease has undergone no significant change throughout western history In North America it remains the serious and unsolved problem it doubtless was long ago in ancient lands On the other hand, Australia has successfully excluded it by quarantine, evidence that there is no hidden reservoir of infection there In New York, where it returns annually from forested areas, it is thought to persist in wild animals and great efforts and energy or much better methods would be required to stamp it out under such circumstances Moreover, the problem is regional rather than one of the State alone New York has succeeded, however, in preventing the invasion of the Adirondack Forest Preserve by rabies virus, an effort justified by the difficulty of eradicating the disease from such a large wilderness area

The story of rabies is inevitably and properly also the story of Louis Pasteur for the study of rabies was the summit of his great career, and his work encompassed most of what we now know of the disease It was first of all the terror of rabies, unique of all the infections in that once begun it invariably causes death, that first challenged him His interest in rabies and indeed his active investigation of it preceded the famous field trials of anthrax vaccine at Pouilly le Fort Pasteur started with long and fruitless efforts to cultivate the microbe Students may well remember that out of those

efforts, in which, on one occasion, saliva from a fatal human case was inoculated into rabbits, came our first description of the pneumo coccus, for Pasteur's rabbits died within two days and in them he found and described the encapsulated organism that was later proven by others to be the common cause of lobar pneumonia. It is to Pasteur's credit that he was not misled by this finding and, having failed to cultivate the rabies agent as he had others he conceived of growing it in the brains of rabbits Pasteur's experiments included the adaptation and modification of the virus in rabbits, its attenua tion by drying and by adaptation, the realization that the long incubation period of the disease provided an opportunity to protect an exposed person by vaccine, as well as observations that suggest an interference phenomenon Dogs that had received large amounts of virulent virus subcutaneously promptly became refractory to rabies 'I am inclined to think that the virus may be accompanied *by a substance which by impregnating the nervous system*, would make it unsuitable for the culture of the microbe"

The discovery of rabies vaccine proved to be the dramatic climax to Pasteur s career and prompted the founding of Pasteur Institutes in many lands This was Pasteur's solution Generally accepted at that time, its effectiveness has not yet been satisfactorily measured in man and to a degree remains in doubt Nothing better illustrates the importance of careful evaluation and of critically controlled trials At the time the incidence of rabies among persons bitten by rabid dogs was believed to be quite high but the opinion was based on a small and random experience Therefore when after many vaccinations Pasteur reported a mortality of 0 5 per cent among treated individuals, there seemed little reason to doubt the value of rabies vaccine Since that time, no one has dared to withhold treat ment consistently in view of the seriousness of the disease and only those countries with limited medical facilities provide opportunities for us to judge the outcome of untreated wounds from rabid animals Neither there nor here has it been possible to judge accurately the risk by which vaccination might be evaluated The evidence that is available, including comparison of the survival of individuals bitten by the same rabid animal, some having received vaccine and others not, all testifies to the value of vaccine although hardly providing precise evidence

The exceptionally prolonged incubation period of rabies, which Pasteur utilized to immunize after infection, was long thought to depend upon the site of inoculation Bites in the leg were associated with longer intervals than those of the face or neck It seems likely, from experimental work of more recent years, that dose is of greater importance as it is indeed in other virus infections, and that there is an unusual delay in the propagation of rabies virus for various still unknown reasons In most individuals, symptoms follow the bite by from one to three months but an appreciable number have shorter intervals and others as long as six to 12 months

The early symptoms of the disease are generalized malaise with fever and headache and vague sensations at the site of the original wound These are followed by signs of a more severe character The classical sign, hydrophobia, is common An attempt to swallow or the thought of swallowing sets off spasm of the throat and patients do not try to swallow their saliva or to drink Stupor or excitement occurs delusions or hallucinations are common Delirium or coma and death follow in a few days or progressive paralysis leads to *exitus*

Fatal human rabies is uncommon in North America and the diagnosis is seldom considered in the absence of a history of exposure Tetanus is probably the most confusing alternative for it too causes spasms which are characteristically more constant than those of rabies Sensations about the original injury may recur in tetanus as well as in rabies Special difficulties arise in persons who develop paralyses following rabies immunization and *poliomyelitis* is a common alternative diagnosis in the paralytic cases

Diagnosis, whether of human or animal rabies rests chiefly on the recognition of Negri bodies, highly characteristic cytoplasmic inclusion bodies of neurons at the cerebral cortex and cerebellum as well as nerve nuclei Ammon's horn is a most likely place to find them They stain intensely red with fuchsin, have a central granular mass of basophilic nature, and may be recognized with confidence by the experienced The Negri body presumably represents a "colony" of virus elementary bodies These themselves are but 100 to 150 mμ in diameter The Negri bodies' staining reaction and appearance are in part due to cellular constituents which form about the mass of virus particles

A more delicate test for the presence of rabies virus is the intra cerebral inoculation of mice with a suspension of nervous tissue and the identification of Negri bodies in animals that succumb usually during the second week following inoculation If the illness is prolonged the isolation of virus may be uncertain

Immunization against rabies has two applications the vaccination of exposed persons and the widespread immunization of dogs in areas where rabies occurs

Two types of vaccine are used One is living but modified virus Pasteur discovered that field strains become modified following prolonged passages in rabbits (virus fixe) Such virus is probably less virulent for man and dogs than the fresh strains but is more virulent for rabbits Interestingly it induces the formation of few Negri bodies Virus long passaged through eggs represents a further stage of modification It does not cause the formation of Negri bodies and is of low rabbit pathogenicity It causes only a silent infection in dogs and is widely used to immunize them The practice is being followed with great interest for the safety of living virus vaccines by their nature more effective immunizing agents than killed virus is something we need to know much more about

Unfortunately laboratory tests of the safety of such preparations cannot be completely relied upon The numbers of animals involved in the field are so much larger than those that may be tested in the laboratory and in the field many variations of possible biologic significance changes in the health and age of the animals as well as breeds may alter their susceptibility and affect the nature of an infection with a modified virus Moreover adapted viruses may not represent pure strains but rather mixed populations of which aviru lent forms simply predominate and the possibility that a virulent virus may arise from such a mixture is a persistent hazard On the other hand it seems well established that the original method of Pasteur which is still widely used does definitely employ the use of small doses of living but modified virus Viruses completely inacti vated by treatment with chemicals such as phenol or with ultra violet irradiation are available that do not so far as we can determine contain living virus

A conclusive comparison of the value of vaccines of these general types and especially of more concentrated but wholly inactivated

virus grown in tissue culture has yet to be made and the preparation of choice is not at present determined

Antirabies treatment is itself of considerable danger and should never be undertaken without ample justification Thus indirect con tact or licks either by healthy or rabid animals are not sufficient justification for the administration of vaccine and only if a dog has symptoms of rabies or is proven rabid should licks on abraded skin lead to the administration of vaccine If the animal s symptoms dis appear and after several days he appears to be healthy, the treatment should be stopped On the other hand, if the rabid animal escapes or is killed, treatment should be begun immediately Based on animal experiments the local treatment of wounds requires no more than thorough washing with soap and water, and the use of strong cauteri zing agents apparently serves no useful purpose In addition, it is worth noting that potent antisera are now available for the preven tion of rabies Antiserum is on occasion used as a substitute for rabies vaccine especially in individuals who have received vaccine on previous occasions While the true value of serum is not well estab- lished, its use preceding vaccination may be life saving in human cases in which the severity of the wounds and their location in dangerous areas (head and neck) suggest that vaccine alone may not be adequate

Avianized virus and tissue culture viruses may be expected to eliminate one of the more serious, if infrequent, complications of antirabies treatment This complication is the occurrence of paralysis shortly after the administration of vaccine Paralysis in these cases is not due to the action of rabies virus but to a degenerative demy elinating lesion of the central nervous system that is due to the repeated injection of brain tissue It is believed that antibodies develop to brain tissue and the reaction of them with antigen gives rise to the lesions which are readily reproducible experimentally It may be seen therefore that the problems of immunization against rabies remain despite the progress that has been made in recent years and that a satisfactory control of the disease itself, the elimination of it, is far from being achieved Until that time, rabies will remain one of the more important virus diseases despite its low incidence

Egg adapted rabies virus, the Flury strain, provides an effective vaccine for dogs and has been widely used Comparative tests indi

cate that the immunity following living virus vaccine persists longer than that produced by killed virus However, the modified virus of course multiplies in the host and while there is no evidence that vaccinated animals are infectious, instances are known in which dogs have died following vaccination under circumstances that suggest the vaccine virus was responsible A certain amount of anxiety is bound to persist in the case of all living virus vaccines until long and varied experience conclusively demonstrate their safety The nature of rabies requires us to be as careful as possible

IX

THE VIRUS LABORATORY SUGGESTIONS REGARDING FACILITIES, EQUIPMENT, AND METHODS

THE FIRST decision in planning a virus laboratory should be the scope and nature of the work This will depend on local conditions In a specialized hospital particular problems may recommend themselves for study The prevalence of certain virus diseases may determine New York State would seem a poor place to study the arthropod borne encephalitides since cases are very uncommon It is usually wise to select problems that are largely unsolved and provide many opportunities for investigation There are doubtless circumstances in which serologic tests alone will be useful in meeting a local problem but there are few rewards from such a program for the pathologist or bacteriologist who is curious about the virus diseases and few opportunities for study If complement fixation tests alone are undertaken the requirements are simple enough Some antigens may be purchased and the required sera prepared in animals or collected and husbanded from local cases Tissue culture technics afford a fundamental laboratory discipline and a basic tool in the diagnosis of virus infections Much may already be done by means of tissue culture alone including diagnostic tests for such significant diseases as poliomyelitis and herpes and the method promises to find additional applications It is most economical of space and animals but it is not now nor ever likely to become a complete substitute for animal experimentation It already is a most valuable auxiliary The experimental animal is more versatile From the infected animal one can draw clues from the symptoms from the tissues after death and from the immune and convalescent responses It would be difficult to proceed without animals and the virus laboratory no matter how small will be fortunate if it can make provision for them The facilities may be quite simple

It is important only that the animals may be adequately segregated from each other and from the staff A hospital laboratory should be especially vigilant in this regard The problem is to prevent cross infections within the unit which can cause errors and the exten

sion of infection outside the unit to hospital patients or workers Both are very real dangers and call for sound planning and technics

The essentials of a satisfactory arrangement are embodied in the the plan of the virus unit of the New York State laboratory The arrangement provides for the isolation of the work with highly infectious agents and the segregation and handling of the various diseases in individual, isolated rooms The individual units consist of a small cubicle for inoculation of eggs and animals, dissections,

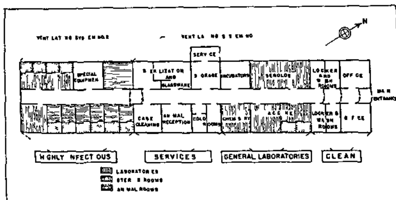


FIGURE 1 Plan of Laboratories for Virology Division of Laboratories and Research New York State Department of Health Albany

etc, adjoining a somewhat larger room for the animal cages A slow, forced movement of air ventilates the entire unit, the flow being from the corridors to vents in the outside walls The highly infectious section has an air supply independent of that of the main unit The two are separated by an air lock in which fresh air is introduced at a greater pressure than that in the two sections of the building and thus prevents them from mixing Similar plans have been used elsewhere with equal satisfaction The principle may readily be adapted to other spaces It is not difficult as a rule to provide for the out of-door exhaust of rooms subject to infection In some cases it may be necessary to disinfect the exhaust either by heat or ultraviolet irradiation If the exhaust cannot be conducted to a remote vent, this should be considered if the more dangerous viruses are to be introduced into the laboratory

Another satisfactory and much more compact arrangement for isolating infected animals was designed by Horsfall and Bauer (*J Bact*, *40* 569 580, 1940) It consists of small cubicles that ac

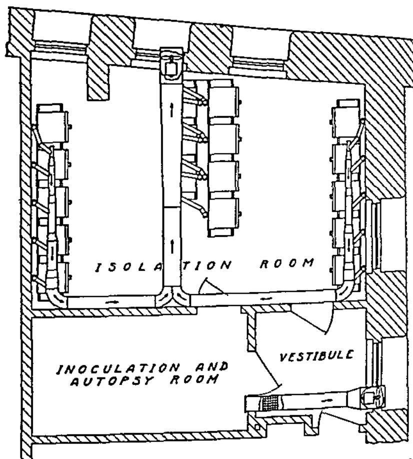


FIGURE 2 The isolation unit designed by Horsfall and Bauer that has proven to be highly successful (*J Bact* *40* 573 1940 Fig 3)

commodate one or more small cages, each cubicle being individually vented by means of a duct The design has been proven effective and should be especially suitable for small laboratories that intend to work with a variety of viruses

Adequate provision for the sterilization of cages is essential and requires access to a large autoclave A rectangular sterilizer two feet

square and four feet deep with monel clad interior is the smallest that can be considered adequate A hospital mattress sterilizer is an ideal size Cages should be autoclaved as a rule before cleaning and whenever the animals are changed It is also important to provide for the safe disposal of infected cage litter, dead animals, and eggs A small incinerator within the unit is desirable and an adequate one can be constructed by a competent mason in a few days if a flue is available Hard woods are ideal fuel for small incinerators, hickory and oak provide the high temperatures required for efficient incineration

Equipment

Small scissors of the kind called cataract or iris scissors equally delicate forceps, and a few heavier scissors, forceps, pushpins, small autopsy boards of soft wood, and sundry cutting tools are needed Syringes from 1/4 to 20 ml size, the larger of the locking type, are necessary Several needle sizes from 1 1/2", 19 gauge for bleeding, and 1/4" or 3/8", 26-gauge for intracerebral inoculation should be stocked The Luer Lok syringes accommodate a convenient little Seitz type filter (Swinny filter) which serves for the filtration of small samples Bacterial filters are now infrequently used because bacterial control by antibiotics is more convenient and simpler, as well as more effective Bacterial filters entrap considerable amounts of even the smaller viruses Nevertheless, filtration must be used at times and the Swinny filter serves well Alundum is used in grinding tissues, and mortars, three to four inches in diameter, are in constant demand Purchase at least a dozen

A conventional centrifuge is useful in clarifying suspensions although most will settle out on standing for a short time If funds are available, an angle centrifuge using one of the newer, high speed electric motors and operating in the range of 8 000 to 10,000 r p m will be a good investment For higher speed centrifugation, the elegant little preparatory Spinco centrifuge should be chosen It is capable of sedimenting even the small viruses, will handle considerable amounts of starting material, is refrigerated (permitting long runs), and extremely simple to operate This type of equipment is not, however, essential to diagnostic work

A refrigerator of good size (it will not be large enough) and a

deep-freezer are essential For the successful preservation of viruses the temperature must be maintained below —20°C A storage box built with separate compartments for solid CO_2 and specimens is better but more expensive to operate Horsfall (*J Bact*, *40* 559 568 1940) designed an excellent cabinet in which the end compartments contain dry ice and the center has tall racks for the systematic filing

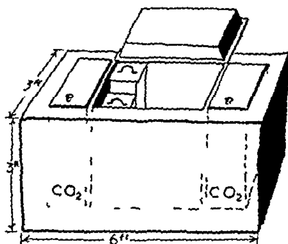


FIGURE 3 The box designed by Horsfall has two compartments for dry ice which *are completely separated from* the central storage section They must be vented to the room The arrangement avoids saturating the storage space with carbon dioxide and makes it unnecessary that specimens be sealed The size shown is practical The end compartments accommodate the usual cakes of dry ice without trimming and the storage space will accommodate a great many specimens if they are well arranged. (*J Bact*, *40* 559 568 1940)

of the specimens It provides for the optimal storage of most material and avoids the necessity of sealing each container against the CO_2 which is necessary if the CO_2 is in contact with the specimens The lower temperature of CO_2 boxes is advantageous in the case of some viruses The deep freezer will serve for others and will be extremely useful in preserving serum samples Serum antibodies are remarkably stable at 4°C and sera may be satisfactorily stored in the refriger ator for many years without loss of activity but the danger of con tamination by molds is reduced by frozen storage

A small lyophilizing apparatus will be useful although frozen storage is in most instances superior to freeze-dry storage A com

pletely satisfactory freeze-dry apparatus can be built about a liter Dewar flask (used to contain the alcohol dry ice mixture) and a heavy Pyrex flask with a side arm The side arm, located near the shoulder of the flask, is connected to a vacuum pump, the specimens to be dried are attached by tubing to the neck of the flask Detailed specifications of a small drying apparatus were given by van Rooyen and Janes (*J Lab & Clin Med*, *43* 489 494, 1954)

Animal cages

Small animal cages represent a considerable part of the cost of establishing a virus laboratory They will be needed in good numbers and, since they require frequent sterilization, should be of non rusting metal We use mouse cages with a double top, one covered with fine fly screening and the lower with quarter inch mesh wire Infected particles that are raised from the bedding do not freely pass through a double layer of screening and flies are prevented from entering the cages The boxes we use are made of 0 025" thick monel and are 7½ × 6¾ × 5" high The top edges are turned down ½" on the inside and soldered The covers are an interchangeable drop-on type, loosely fitted, and made in two separate units The top section is covered with 16 × 16 monel screen, the bottom has 3 × 3 monel screen The top frames are of the same stock as the box itself The covers are reinforced with corner plates All joints are soldered or welded Specify a 7/16" hole in the corner for the water bottle Pyrex glass jars may be considered They are the cheapest and in many ways very satisfactory With careful workers they might well be the first choice

Cages of a somewhat larger size are used for guinea pigs and hamsters These should be equal in quality to the mouse boxes The dimensions may be 8 × 15 × 6¾" high Double screening is again desirable

Animals

A satisfactory supply of disease-free test animals is, of course, essential and, if it is possible to maintain a mouse colony, this should be done (Farris, E J, ed *The Care and Breeding of Laboratory Animals* New York, Wiley, 1950) If the animals are purchased, try to provide for uniformity in strain of animals and source of

supply Age and size are important It is good practice to test the mouse colony for the presence of latent lymphocytic choriomeningitis virus This can be done by harvesting a mouse brain and inoculating a number of the stock mice intracerebrally with 0 03 ml of a 10 per cent suspension of the brain If latent choriomeningitis virus infection is present, the disease will become manifest in the test mice Albino mice are as a rule carriers of Theiler's mouse encephalomyelitis virus It is possible to maintain a colony free of Theiler virus infection although strict isolation and rigid control of the sterility of food and bedding is necessary to prevent infection The presence of Theiler virus in a colony is readily determined by testing intracerebrally in mice a fecal specimen collected from young adult animals There is no evidence that latent infection with this agent significantly interferes with the susceptibility of albino mice to the known human viruses

Mouse colonies have at times been found to be latently infected with several other viruses (Staff of the Roscoe B Jackson Memorial Laboratory, *Biology of the Laboratory Mouse* Philadelphia, Blakiston, 1941) One agent that has recently attracted attention causes widespread necrosis of the brains and livers of mice, suckling animals being especially susceptible (Cheever, F S, *et al J Exper Med*, *90* 181 194, *195-212*, *1949*, Nelson, J B *J Exper Med*, *96* 293 302, *303 312*, *1952*, Morris, J A, and Aulisio, C G *Federation Proc*, *13* 506, 1954) The hepatic lesions characteristically consist of multiple foci of opaque, grey nature composed of inflammatory cells The cellular changes are suggestive of virus infection Mouse pox or ectromelia is another disease to bear in mind The circumstances attending an outbreak of ectromelia in a mouse colony used for virus studies might profitably be consulted (Melnick, J L, and Gaylord, W H Jr *Proc Soc Exper Biol & Med*, *83* 315 318, 1953) A third is PVM or pneumonia virus of mice (Horsfall, F L, Jr, and Hahn, R G *J Exper Med*, *71* 391 408, 1940) which induces a lesion resembling influenza in the lungs of mice intranasally inoculated with mouse lung suspensions Repeated blind passages have revealed transmissible strains of PVM virus in two-thirds of laboratory mice These natural infections of mice might well be studied as an introduction to work with human pathogens Latent virus infections in the test animals must always be kept in mind and

the experimenter will be wise if he controls and standardizes his tools as much as he can The more he knows of his test animals and reagents, the more he can rely on his results

Egg incubators

Provision must be made for both primary and secondary incubation of fertile eggs and there are few places in the country where a good source of fertile eggs cannot be found The Jamesway incubator No 252 (James Manufacturing Company, Elmira, New York) is entirely satisfactory for primary incubation and smaller inexpensive units will be needed for the secondary incubation of infected eggs The American Lincoln Incubator Company, 645 Somerset Street, New Brunswick, New Jersey, manufactures a 600 egg unit, lined with stainless steel, that is excellent for the purpose

Few instruments are required A good egg candler, one with an intense light, is needed, syringes and several heavy, sharply pointed probes All of the egg technics can be carried through with the use of a probe and syringes Eggs to be inoculated can best be stood in a twisted ring of cotton wet with lysol and placed in a Petri dish Rings of this kind are easily prepared as needed and then discarded

Tissue culture

Satisfactory tissue culture work may be accomplished in an ordinary bacteriologic incubator Install a maximum minimum thermometer as a check on the sensitivity and reliability of the thermostat Provision should be made, as through the use of small trays for slanting the tissue culture tubes sufficiently to prevent the fluid from coming in contact with the neck of the tube If roller tube technics are used, a suitable drum may be constructed of light material in which the tubes can be mounted Such a drum is readily rotated by means of one of the small slow motors manufactured for electric clocks by Telechron Department of General Electric Company, Ashland Massachusetts

Histologic laboratory

It is a great advantage to have access to a good histologic laboratory so that tissue specimens can be promptly prepared for microscopic examination Histologic examinations are much neglected by

virologists but can be of the greatest value in revealing the nature of infections and frequently provide important clues Histologic examination should not be perfunctory or routine, only selected tissues and experiments deserve microscopic study But a supply of Bouin s solution for the fixation of specimens should always be on hand and specimens taken whenever the behavior of the animals or the appearance of their organs suggests an unexplained condition

It is probably unnecessary to note that electron microscopy is rapidly opening new vistas in morphology and that the intracellular lesions that are characteristic of virus infections, as well as the visualization of the viruses themselves, provide countless fascinating opportunities for investigation But electron microscopy belongs to all of morphology and is not only a virus technic

Reagents

One indispensable solution is a mixture of penicillin and streptomycin (500 U of the former and 2 5 mg of the latter per ml of test material) for the treatment of specimens that are contaminated with bacteria The antibiotics have made it possible to salvage many specimens and a stock solution of penicillin and streptomycin should be kept on hand at all times Normal rabbit and horse serum should be available Specific antisera for the agents under study are required and should be prepared by the investigator The antisera should be prepared in sufficient quantity to permit their thorough standardization and evaluation It is a great advantage to have ample supplies of thoroughly studied antisera Antigens for use in complement fixation tests may be prepared by the investigator or purchased Viruses that may be encountered or with which the worker intends to experiment must be collected and maintained The method of preserving them varies but all may be safely held in storage with dry ice Viruses may be purchased from the American Type Culture Collection, 2029 M Street, N W , Washington 6, D C They may frequently be obtained from other investigators It is most important that the origin and identity of strains be known with certainty

A few chickens will be needed if chicken red blood cells are to be used In many cases human type O red blood cells can be substituted Sheep red blood cells are required for certain hemagglutination tests as well as for complement fixation tests

Library

It is obviously of great importance to the investigator to have access to adequate library facilities A number of books will be consulted so frequently that they belong in the laboratory *Diagnostic Procedures for Virus and Rickettsial Diseases,* 2d ed American Public Health Association, New York (in press), is a useful reference *Viral and Rickettsial Infections of Man* (Rivers, Thomas M, ed, 2d ed Philadelphia, Lippincott, 1952) and the *Textbook of Virology* (Rhodes, A J, and van Rooyen, C E 2d ed Baltimore, Williams and Wilkins, 1953) are useful The investigator familiar with French will find much of value in the comparable work by Lépine and Soulier (*Techniques de Laboratoire Appliquées au Diagnostic des Maladies à Virus* Paris, Masson, 1954), the *Handbuch der Virusforschung* by Doerr (Wien, J Springer, 1938 1950, 4 vols) has many excellent sections The periodicals of first choice would include the *Journal of Experimental Medicine, Proceedings of the Society for Experimental Biology and Medicine, Journal of Immunology, Zeitschrift fur Virusforschung,* and the *Bulletin of Hygiene* The last may be purchased unbound and printed on a single side of the page In this form it is suitable for clipping and the preparation of an abstract card file

X

THE SIMPLE COMMON TECHNICS

IN WRITING this little introduction to virology, the author has had hospital pathologists much in mind for it is his experience that hospital pathologists have unusual incentives and opportunities for the study of virus diseases—not, perhaps, so much in the investigation of a particular problem or disease as in the more expert and precise diagnosis of the various infections that come to their attention Hospitals and hospital pathologists are busy with the care of the sick, their work is an endless succession of frequently puzzling and usually atypical cases Each may become a pressing problem because of the human values involved The tempo of hospital work and the problems of individual disease tend to make the hospital pathologist a pragmatic fellow but his scientific background and tastes drive him to meet these problems in a scientific manner and to learn more and more about the diseases with which he copes He is uniquely fitted, therefore, by background and circumstances, to investigate human virus infections

His successes and satisfactions will depend on many factors of which none is more important than the attention and skill he devotes to the collection of *good* specimens Here he may excel if he wishes for he has the best chance of all to collect the specimens he wants Judgment and care are needed Judgment is necessary to select the cases that deserve special study and to collect the relevant specimens Judgment is needed to select the specimens that may be interesting for special reasons or that may afford an opportunity to learn something new Nothing is possible without a suitable specimen With excellent starting material most searches prove to be surprisingly simple Thus much hinges on getting the right specimen Karl Meyer has told how he isolated the virus of equine encephalomyelitis, which, in turn, led to a series of discoveries of new agents during the 1930's Meyer had been asked to investigate an outbreak of a mysterious fatal disease of horses in California He had sought by various means and without success to isolate an infectious agent from the brains of dead horses and realized that he required better,

in this case earlier specimens Tramping through the countryside he finally found a sick horse and through boldness and persistence managed to buy and destroy it and collect the brain for study It was from this animal that the first isolation of equine encephalomyelitis virus was made The isolation had depended not on method but on the quality of the specimen The hospital virologist should develop the habit of spending as much time as possible on the wards and in the clinics and in conversation with his colleagues who will most likely know of instructive cases He should realize that it is more important to spend his effort on the collecting of suitable starting materials than in trying to cultivate an agent from unsuitable ones The rule should be to collect samples first and on small suspicion and then to postpone the testing of them until it is evident that the best available material is at hand Acute and convalescent phase sera which are often so necessary to prove virus infection should likewise be collected on the slightest provocation It is especially important that the initial serum be harvested early in the course of the disease and it is good practice to take double the amount of blood that is customarily sent to the laboratory for other serologic tests that is two rather than one tube of blood

It is good practice for the pathologist to be prepared to collect specimens properly in the post mortem room A collection of small instruments should be on hand that can be quickly sterilized and used for the harvesting of interesting material The level of bacterial contamination in post mortem rooms is very high and most specimens collected in them and sent to virus laboratories for examination are unsatisfactory because of soiling that could easily have been avoided Remember that the specimen may be contaminated with a virus as well as bacteria if the collection is not properly made If material is to be taken for inoculation of animals eggs or tissue culture the procedure should be planned in advance so that the specimens can be collected before the tissues are badly contaminated If the decision to collect specimens comes as an afterthought the larger surfaces of organs had best be seared with a red hot spatula and freshly exposed tissues harvested with sterile tools If the specimens cannot be frozen they had best be stored in 50 per cent glycerol (C P) which has the added advantage in the case of autopsy specimens of being bactericidal Storage in glycerol works well for many viruses

especially so for the neurotropic viruses and influenza Poliomyelitis virus is stable in glycerol for many years

Materials that are to be inoculated into animals or eggs or tissue culture should be promptly frozen and held at low temperatures until prepared for injection The samples should be of sufficient size so that enough is left to repeat the isolation if there is any doubt of its validity Large thermos flasks containing dry ice or cracked ice are convenient for carrying specimens in the field and for their temporary storage but a carton well insulated, as with crushed paper wadding, will serve The serum is separated as usual from the blood clot, cultured on ordinary media, and may be stored in the ordinary refrigerator or frozen It is advisable to dispense the serum in 1 0-ml amounts in tightly sealed tubes *Do not freeze whole blood* unless it is to be used for virus isolation for hemolyzed serum is useless in certain serologic tests

Specimens that might be infectious should be handled with appropriate care Since frozen materials are difficult to withdraw from small necked bottles, it is helpful to use a straight sided, screw capped jar of small size (one ounce) This should be filled no more than half full, be properly identified, and placed in an uncontaminated outer case Lusteroid tubes are frequently used, chiefly because they do not crack when frozen They are, however, difficult to sterilize and are highly inflammable Specimens may be easily contaminated on the ward unless proper precautions are taken The use of previously sterilized disposable paper plates and cups and of wooden tongue depressors is practicable

A register of all specimens received is desirable The number assigned each specimen provides double assurance of the identity of the material

In preparing infectious specimens for testing or otherwise handling them, it is well to cover the workspace with a towel previously soaked in 2 per cent cresol and wrung out and spread over the space in front of the worker The wet towel prevents dust from flying and absorbs and dilutes droplets of infected material Such towels, a good grade huck toweling is advised, should be routinely sterilized in the autoclave A number can be stored in an enamel bucket Cresol has very little virucidal activity but is, of course, bactericidal

Plastic eye shields (No 1120-C, C H Dockson Company, De-

troit, Michigan) or regular spectacles and masks, gowns, and caps should be worn when dealing with dangerous material, thin rubber gloves are at times desirable

Vaccination of the staff should not be neglected if dangerous agents are introduced into the laboratory No one should work with the agent of Q fever or the encephalitides without previous immunization

Preparing suspensions

Solid or particulate specimens must be ground and clarified to allow injection Half teaspoonful amounts of alundum can be sterilized in test tubes and kept available for grinding Porcelain mortars and pestles are sterilized in separate paper bags and kept on hand The specimen, whether tissue, a cotton applicator, or semisolid material such as feces, is placed in the mortar, weighed if possible, the alundum added, and the mixture ground thoroughly, adding diluent with a pipette to make a suspension of approximately estimable proportions Either physiologic saline (0 85 per cent) alone or physiologic saline containing 10 per cent beef infusion broth (broth salt solution) makes a suitable diluent If the suspensions are to be stored as stock test material, it is well to fortify the diluent with a little sterile milk or normal serum (rabbit or horse serum) in 2 to 5 per cent concentrations after first ascertaining that it has no deleterious effect on the material for which it is to be used Viruses are more stable when stored in this way

The supernatant may be decanted into a suitable centrifuge tube, clarified by spinning in the bucket type centrifuge for 5 to 15 minutes at speeds of from 1,500 to 3,500 r p m depending on the nature of the sediment The final supernate should then be decanted into a small wide-necked glass jar with screw top of the kind recommended for storing specimens These small jars have tight closure, stand upright on the table, and are cheap

The suspension should be streaked on a blood agar plate which is incubated overnight and examined for evidence of bacterial contaminants If no growth occurs, the material may be used as is although, since this is a very crude test, it may not be sterile Otherwise the mixture of penicillin and streptomycin (500 U penicillin and 2 5 mg streptomycin per ml is satisfactory for fecal specimens) is added

Alternative methods are to allow the specimen to stand in mixture with ether overnight, remove the ether by evaporation the following morning, and culture Two runs in the centrifuge at maximum speed (3,500 r p m) for periods of 30 minutes are usually effective in sedimenting bacteria from saline suspensions It is especially important that suspensions be bacteriologically sterile before they are tested in eggs or in tissue culture

Antisera

A number of factors enter into the preparation of antisera It is desirable to immunize animals of sufficient size to permit a reasonably large bleeding If rabbits respond to the antigen, they are suitable Rabbits are not very susceptible to many human virus diseases and may be injected directly with living virus, intraperitoneal inoculations being repeated at weekly intervals using 0 5 to 1 0 ml amounts of antigen In some cases, to produce potent antiserum it may be necessary to give a fourth injection of at least 1 ml intravenously Bleedings are usually taken one week after the last injection

If the animals are susceptible to the virus, the immunization procedure must begin with very small doses or with high dilutions or with virus that has been inactivated with formalin Frequently a strain of virus is available that is of low pathogenicity for the animal in question Moreover, there are often opportunities to collect immune serum from test animals, from the sick but surviving animals in a virus titration or neutralization test During the isolation of a virus, early passages in mice may result in only a few deaths but the test animals may nevertheless have become immune Their sera can be especially useful in establishing that the virus was present in the early passages and therefore more likely was in the original specimen

Inoculation of animals

A choice must be made regarding the species of test animals, their age and route of inoculation In general, younger animals are more susceptible There are two noteworthy exceptions Poliomyelitis virus (Type II) and freshly isolated strains of lymphocytic choriomeningitis virus are *less* pathogenic for infant mice Other common viruses are more pathogenic for infant mice, and, for certain Group *B Cox*

sackie virus strains, it is preferable that the animals be no more than one day of age In general, it is well to use mice two to three days old since the newly born are less able to withstand the traumatic effects of inoculation and are more apt to be eaten by their mothers It is better to use mice of various ages and more than a single route of inoculation (but only one route per individual animal) unless the nature of the virus is strongly indicated by circumstances In general, neurotropic viruses are injected intracerebrally

Guinea pigs are sometimes useful especially since the effects of inoculation may be checked by daily rectal temperature measurements In this case, keep separate thermometers for each test animal Rabbits have limited value in the isolation of viruses Intracerebral inoculations should be avoided as domestic rabbits are at times carriers of herpes virus and intracerebral inoculation is likely to activate such an infection This has been a notorious cause of error in the past

A hamster colony is easily maintained and, while the rate of multiplication is much reduced during the winter, the animals are infrequently troubled by intercurrent infections and are surprisingly disease-free Newborn hamsters can usually be used in place of baby mice The hamster has no unique advantages although it is quantitatively better than the mouse as a source of antiserum

Inoculation of eggs

Three routes of inoculation are commonly used, the yolk sac and the allantoic and amniotic cavities Amniotic inoculation directly infects the respiratory tract The yolk sac is equivalent to an intravenous inoculation because of its great vascularity Embryos 5 to 8 days old should be used since the yolk sac is proportionately larger at that time Inoculation is made through the air sac with a 1½ inch needle after puncturing the shell previously wiped with iodine, with a sharpened probe The allantoic sac may be entered in the same way or through a second opening, over the embryo and between blood vessels Embryos 10 or 11 days of age are suitable The amniotic sac is the route used chiefly for the isolation of strains of influenza virus The air sac must be punctured, a small opening made in the side wall of the shell adjoining the embryo (as located by candling), the shell membrane fibers separated, and the chorio allantoic membrane dropped by sucking on the hole in the air sac

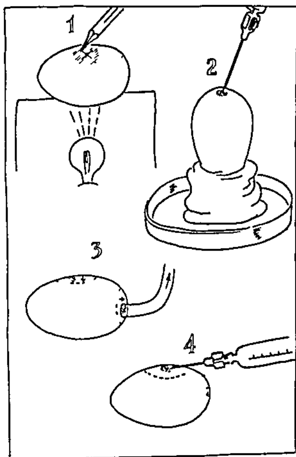


FIGURE 4 (1) As a first step identify the position of the embryo by a pencil mark on the shell so that it can be avoided For yolk or allantoic sac inoculations the large end of the egg is stabbed with a ¼" needle and the injection made forthwith. The air sac will have been punctured incidentally and provides sufficient displacement for the inoculum (2) Inoculation of the chorioallantoic membrane requires that two small defects be made in the lateral shell and shell membrane (3), and into the air sac By aspirating with a section of rubber tubing or a bulb, the membrane can be dropped to create a new, artificial air sac, the basement membrane of which is chorioallantoic membrane rather than the shell membrane which lines the true air sac. The inoculum is then dropped onto the membrane by means of a syringe or capillary which should be held in a nearly horizontal position to avoid traumatizing the membrane (4)

with a rubber bulb Once the membrane has dropped, the shell may be opened more widely and the embryo brought under direct observation. The amniotic membrane can then be identified and the injection made within the sac.

A similar technic serves for infecting the chorioallantoic mem-

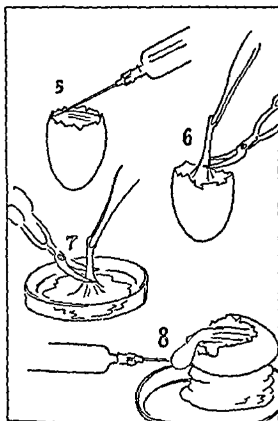


FIGURE 5 Harvesting of egg material is equally simple The allantoic fluid can be aspirated once the shell over the air sac has been removed If the needle opening is directed toward the shell, the membranes can be avoided (5) Yolk sac is collected either by lifting it from the opened shell or breaking the egg into a Petri dish and then collecting the membrane (6 and 7) Amniotic fluid is easily harvested after opening the shell by gently shifting the contents until the sac overflows the shell edge The thin wall and clear fluid are distinctive and the fluid may be aspirated into a syringe (8)

brane The membranes are dropped and the inoculum distributed over the surface with a syringe and needle The shell openings can be closed with an inch wide strip of Scotch tape The curvature of the egg makes it possible to fold the ends of the strip together forming two handles that are useful when the shell is to be more widely removed Scotch tape also holds bits of shell during harvest Melted paraffin is often used to close the smaller openings in the shells

Chicken embryos may be inoculated intravenously with a blunted bevel needle and veins of the membranes or the embryo itself, its brain or peritoneal cavity, may be entered These methods are seldom necessary

Tissue culture technics

Very rapid progress is being made in the cultivation of viruses in tissue explants and cells grown *in vitro* An endless variety of technics is available and it behooves the beginner to choose a method best suited to his situation and problems and stick to it until it is thoroughly mastered The hospital pathologist has the advantage of seeing the human tissues removed surgically From these he can select samples suitable for tissue culture Human uterus, for example, supports the growth of all three types of poliomyelitis virus and can be used for serologic tests with all of these Human embryonic tissue may become available at times and has unique value in tissue culture study of human viruses because it has the growth capacity of embryonic cells as well as the advantage of its human origin At least one strain of human cancer cells is available for tissue culture work that may be maintained as a perpetual source of culture cells in the laboratory (HeLa cells)

Tissue culture technics follow one of several patterns The bits of tissue are cultivated in small flasks or tubes with a nutrient medium (satisfactory synthetic media are now available and may be purchased) plus serum and a little embryo extract or the explants may be stuck to the walls of tubes or cover slips mounted on hollow ground slides by means of plasma clot, fiber glass cloth or other means The nutrient medium is replaced periodically The transplanted cells themselves may be used to support the growth of virus or outgrowths from them may be essential In the latter case a pre

liminary incubation period is required before the culture may be used

Virus growth may be recognized by necrosis of the cells, but this does not always occur In the absence of such an effect, tissue cul ture becomes involved and much less satisfactory It is possible that the cytologic changes that do occur may prove to be more charac teristic than they now seem to be and thus provide clues to identity Much more remains to be learned about these changes

It is evident that diagnosis of virus infections may in some cases be made by tissue culture methods either by isolating a virus or by serologic tests in which viruses on hand are tested against various dilutions of serum by means of tissue cultures These are the meth ods of choice in the case of poliomyelitis at present

Where small inexpensive animals are known to be susceptible they obviously offer a number of advantages over tissue culture Animals are simpler to maintain, are more rugged hosts, and in their responses disclose more of the nature of their infections Certainly provision should first be made for animal experimentation Tissue culture should be introduced when and if possible, but only as a supplemen tary technic Much time and effort will be saved if the worker can arrange for personal instruction in an established laboratory

The neutralization test

This is the basic serologic procedure in virology and yields by and large the most specific and significant evidence of the antigenic iden tity of viruses and of immune responses to infection The presence of a virus in many cases, or its apparent recovery from a patient, may be open to various interpretations, since the subject may have been only a carrier or the virus may have been picked up when the speci men was collected or during handling in the laboratory It is there fore often necessary to demonstrate an immune response in the pa tient For this purpose before and after, or at least acute and con- valescent, blood serum specimens are needed In individual cases such samples can usually be most simply and conclusively tested for their ability to neutralize the virus in question

The neutralization test requires that the two sera be compared for their ability to "neutralize" the virus, that is, to prevent infec

tion, using eggs, animals or tissue culture as the *criterion of infection* Specific neutralizing or positive sera prevent homologous in fection Quantitative comparisons are usually necessary and always desirable To begin with, one needs to know the titer of the virus suspension being used in the test This is determined by making serial dilutions of the original suspension beyond the *dilution that* yields 50 per cent deaths or whatever other manifestation of infec tion is chosen as the criterion When that is known, equal parts of broth salt (for control) and serum and of each dilution of virus sus pension (a further 1 1 dilution of the virus) are mixed in small tubes and incubated for an hour at room temperature and injected into groups of test animals, eggs, or tissue cultures The score is kept as for the titration of the virus and is similarly interpreted

An alternative method is to mix a predetermined number of doses of virus (10 or 100 ED_{50}) with various dilutions of serum again in equal parts The advantages of this procedure lie in economic use of the sera and in greater delicacy of the test Moreover, a greater range of antibody titer can be measured if a constant amount of virus is used Neutralization test results vary in many instances with the route of inoculation Intraperitoneal tests generally are more sensitive and indicate a greater neutralizing range, for example than intracerebral inoculations but require larger inocula For in tracerebral inoculation, 0 02 0 03 ml amounts are usually injected for subcutaneous inoculations, 0 03 ml is *suitable, for the intra* peritoneal route, 0 05 ml is more appropriate These quantities apply to mice

The identification of a virus by means of an immune response is usually self evident If the results are to be expressed numerically, it is necessary to estimate the neutralizing index This like index of virus infectivity, is determined by establishing the dilution that would presumably infect or protect half the mice, the ED_{50} This may be simply estimated by determining the morbidity or mortality rates for dilutions that yield greater and lesser values than 50 per cent and calculating the proportional distance between the two by the formula 50 minus the mortality at the lesser dilution divided by the mortality of the higher dilution minus that of the lower The method of Reed and Muench (*Am J Hyg*, 27 493 497, 1938) is widely used for calculating the titer of viruses and antisera although

more elegant methods are available The moving average method is used in the author's laboratory (Thompson, W R *Bact Rev 11* 115 145, 1947)

Of greater practical importance is the inclusion of suitable con trols A known immune serum as well as a normal serum should

TABLE 2

AN EXAMPLE OF A SATISFACTORY COXSACKIE VIRUS NEUTRALIZATION TEST

Serum							*Results Virus—Mouse Brain Suspension Group A Type 1 No 48249*	
Group	*Type*	*Strain*	*Species*	*Antigen*	*Bleeding Date*	*Dilutions**	10^{-1}*	10[illegible]
		Normal	Mouse		2/14/49	Undiluted		8/8
			Hamster		10 24 49	"		8/8
			Monkey		11/10 49	"		8 8
A	1	48249	Monkey	Hamster leg	6 29/49	1 100	0 8	
						1 1000	1 8	
			Hamster	"	5/19 50	1 100	0 8	
						1 1000	0 8	
				Brain	5/ 5/50	1 10	0 8	
						1 100	1/8	
						1 1000	8 8	
	6	5011	Mouse	Brain	1/26/50	Undiluted		8 8
	7	50140			3/ 9/50	"		8 8
	8	5010			3/22/50	"		8/8

* Original dilutions.

Denominator indicates number of mice inoculated numerator number dead, paralysed or missing during the critical period of the test.

The virus was not neutralized by serum of normal mouse monkey or hamster but was neutralized by such sera following immunization The serum dilutions used permit comparison of the potency of the antisera prepared in hamsters by infected muscle and brain inoculations. The hamster leg suspensions contain significantly larger amounts of virus and induce greater immune responses. No neutralization occurred when Types 6, 7 or 8 antisera were used.

always be included The normal and the test sera should be from animals of the same species if possible The most suitable negative control serum is, of course, an early bleeding from the immunized animal or the individual being tested for humoral antibodies Since some sera contain inhibitors for certain viruses, it is good practice to inactivate all sera at 56°C for 30 minutes in a water bath unless the absence of nonspecific inhibitors has been proven The interpre-

tation of neutralization tests requires some knowledge of the nature of the agent and host since viruses differ greatly in their antigenicity and a titer of antibodies may be considered significant in one case but not in another Thus, while respiratory virus infections may be followed by slight or moderate immune responses that are neverthe less of diagnostic value, equal differences encountered in neurotropic infections might mean very little The plan of the test and especially the suitability of the controls are more important in judging its sig nificance than the precise calculation of the doses neutralized

Few practices are likely to be more instructive than that of in cluding additional sera in neutralization tests even if some are chosen more or less at random The sera from other patients recently recovered from infections or a sample of pooled adult serum col lected in the area perhaps an antisera studied in earlier work may at times yield surprising and highly instructive information Tests limited to the bare essentials imply that the experimenter can fore tell the results of the test and there are few experimenters of whom this is true The golden rule, therefore, is to *experiment* and a good place to do so is in the neutralization test which provides many op portunities for the virus fisherman

Hemagglutination test

Since 1941 when Hirst and McClelland and Hare observed that the virus of influenza caused agglutination of chicken red blood cells, a variety of viruses including mumps, smallpox, some of the enceph alitides and foot and mouth disease has been found to agglutinate the erythrocytes of certain species under certain special conditions The hemagglutination test at present remains the most useful for studies of influenza and mumps in man and of Newcastle disease in poultry, since for these viruses comparatively simple technics and materials are required In the case of other viruses the conditions of the test at present are more exacting and the reactions less sharp and other technics more suitable However, it is quite likely that the range of usefulness of hemagglutination tests will be greatly ex tended in the future Cells sensitized in various ways and indirect procedures that may be used to identify adsorbed antibody rather than viruses offer prospects of inexpensive and simple methods that are well suited to virus diagnosis The hemagglutination test itself

is useful in the presumptive diagnosis of influenza The allantoic fluid of embryonated eggs successfully inoculated with throat washings *from a patient suspected of having influenza will agglutinate chicken* erythrocytes, indicating that a strain of virus has been isolated The *unknown virus may then be identified by treating it with known* influenza antisera The homologous serum inhibits hemagglutination [illegible]

[illegible]

ability to inhibit hemagglutination of homologous virus over that *taken from the same patient early in the disease*

The Hirst method is widely used for influenza studies The necessary *materials include infected allantoic fluid* of known or unknown type, heat inactivated (56°C for 30 minutes) acute and convalescent *sera from patients or* known normal and immune antisera, a freshly prepared 1 5 per cent suspension of 3 times washed chicken red cells and *a supply of 0 85 per cent sodium chloride* Human red cells could be substituted especially since they should be easily obtained in a hospital The *virus and the sera must be handled with sterile* precautions in order to keep them for future use, but the test itself may be done in clean but *not sterile glassware The worker should* take care not to infect himself Many cotton plugged 1 0 ml pipettes graduated in 100ths will be needed, as well as many small 11 by 75 mm tubes selected for uniformity of shape and diameter Long racks that hold at least 12 such tubes in a single row are most useful

Color standards are prepared by making 10-100 per cent dilutions of a 1 5 per cent suspension of red cells in physiologic saline For example, 10 tubes *may be set up with 1 9 ml of saline* and 0 1 ml of red cell suspension in tube No 1 1 8 ml of saline and 0 2 ml of red cells in tube No 2 and so on down to tube No 10 which contains 1 0 ml of each The titer of the virus to be used is determined by the *hemagglutination test* Two fold dilutions of infected allantoic fluid are made in saline with a clean pipette for each dilution An equal amount of 1 5 *per cent* erythrocytes is added to each tube including a control tube containing saline The mixtures are allowed to stand at room temperature for one hour, during which time the agglutinated cells settle to the bottom The titer is determined by

comparing the density of red cells still suspended in the lower third of the tubes with that of the freshly shaken color standards There is said to be one agglutinating dose of virus in the last tube showing 50 per cent agglutination Once the virus titer is known, the *hemagglutination inhibition test* may be done Serial two-fold dilutions of sera are made, again using fresh pipettes for each dilution, and mixed with an equal volume of virus of one dilution The erythrocytes are added as before The virus dilution to be used throughout is calculated as one capable of giving four agglutinating doses after the final addition of two volumes of red cell suspension The end point of the serum is found in the tube that shows 50 per cent agglutination, representing one hemagglutination inhibiting unit A number of unknown sera may be quickly checked in this way against one or more known types of virus, or unknown viruses may be identified (*J Immunol*, *65* 347-353, 1950)

Complement fixation

The quantitative complement fixation test is routinely used in the Division of Laboratories and Research, New York State Department of Health, in the diagnosis of the encephalitides (W E E, E E E, and St Louis encephalitis), herpes simplex, influenza, lymphocytic choriomeningitis, lymphogranuloma venereum, mumps, psittacosis, Q fever, Rocky Mountain spotted fever, typhus, and vaccinia The technic used is similar to that described for syphilis in Standard Methods (Wadsworth, A B *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 1947, 3d ed, Baltimore, Williams and Wilkins, pages 361 465) Two-fold dilutions of serum are tested with three 50 per cent units of complement Fixation for 24 hours at 3° to 6°C is practiced

The optimum dose of antigen is determined as the amount which gives maximum fixation of complement under the conditions of the test (In the example shown in Table 3 the 1 2 dilution of antigen appears to be optimum for three units of complement and with dilutions of serum from 1 4 1 to 1 125) Dilutions of serum are tested with optimum doses of antigen and with varying amounts of complement, using proportionally increasing amounts of antigen and complement for the increasing amounts of serum The amount of com

plement required for 50 per cent hemolysis is determined from the partial hemolysis obtained by means of graphic extrapolation or conversion factors applicable to the system The maximum values obtained with each amount of serum are plotted against the quantities of serum used and a linear relationship will be found between serum and complement From this line the titer can be determined by extrapolation as the number of 50 per cent units of complement

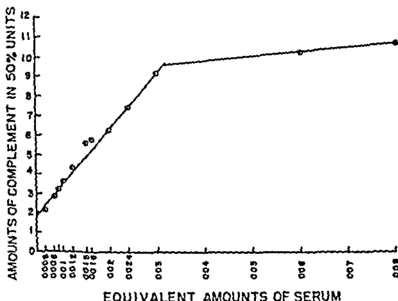


FIGURE 6 Illustration of the linear relationship between serum and complement in a system in which the antigen was St Louis encephalitis virus

required for 50 per cent hemolysis when undiluted serum reacts with antigen in optimum dose.

The data presented in Table 3 have been plotted in Figure 6 to illustrate the linear relationship between serum and complement in the range between 2 and 9 units of complement. After 9 units the slope of the line decreased, signifying that the maximum reactions had not been obtained with the doses of antigen employed and with 0 003, 0 006, or 0 008 ml. of serum.

Four-fold increases between acute and convalescent specimens are usually significant.

TABLE 3

TEST FOR LINEARITY BETWEEN SERUM AND COMPLEMENT RESULTS EXPRESSED IN PER CENT HEMOLYSIS

Antigen	Serum	Serum Dilutions	Equivalent Amt of Serum in 0.05 ml Each Dilution	Amounts of Complement (1) and Antigen Dilutions Used (2)															Maximum 50 Per Cent Units Fixed*
				(1) 3 Units				(1) 4.5 Units			(1) 6 Units			(1) 9 Units		(1) 12 Units			
				(2) Undil	1:2	1:4	1:8	(2) 1:1.33	1:2.67	1:5.33	(2) Undil	1:2	1:4	(2) 1:1.33	1:2.67	(2) Undil	1:2		
			Ml																
St Louis encephalitis virus	Hyperimmune guinea pig frozen	1:4.1	0.012													50	100		12.0
		1:6.25	0.008											35	95	75	100		10.5
		1:8.33	0.006								0	15	75	55	95	80	100		10.1
20-per cent mouse brain suspension irradiated lyophilized		1:12.5	0.004					0	20	70	0	25	80	60	100	100	100		>9.05
		1:16.7	0.003					0	20	80	5	35	95	80	100	100	100		9.05
		1:20.8	0.0024					15	40	85	20	60	95	90	100	100	100		7.4
		1:25	0.002	0	0	0	60	10	40	90	45	65	95	95	100				6.2
		1:31.3	0.0016	5	0	10	65	25	60	95	55	80	100	100	100				5.76
		1:33.3	0.0015	5	5	10	65	35	70	95	60	80	100	100	100				5.59
		1:41	0.0012	20	15	35	80	80	85	95	85	95	100						4.37
		1:50	0.001	35	25	60	80	90	90	95									3.64
		1:55.5	0.0009	50	40	60	85	95	95	100									3.24
		1:62.5	0.0008	70	55	60	90	95	95	100									2.86
		1:83.3	0.0006	90	85	85	90												2.12
		1:100	0.0005	95	93	93	95												
		1:125	0.0004	100	90	90	95												
				Optimum dilution for 3 units 0.1 ml, 1:2				Optimum dilution for 4.5 units 0.1 ml 1:1.33			Optimum dilution for 6 units 0.1 ml undil								

The usual serum antigen complement sensitized cells and normal antigen controls are included in each test
* Unextrapolated values taken from Table 28 in Standard Methods.

TABLE 4

RANGE TEST TO DETERMINE ACTIVITY OF ANTIGEN RESULTS EXPRESSED IN PER CENT HEMOLYSIS TITERS WERE DETERMINED BY USING TABLE 28 FROM STANDARD METHODS

Test Antigen	*Per Cent Hemolysis and Titers with Following Serum Dilutions and 2 Units Complement (0 05 ml each Serum Dilution Used)*											
Dil.	1 2	Titer	1 4	Titer	1 8	Titer	1 16	Titer	1 32	Titer	1 64	Titer
Undil.	0		0	>20	40	25	100		100		100	
1 2	0		0	>20	3	40	100		100		100	
1 4	0		0	>20	5	40	55	46	100		100	
1 8	10	9	0	>20	20	31	70	40	100		100	
1 16	40	6	75	10	100		100		100		100	
1 32	95		100		100		100		100		100	

The technic described by Fulton and Dumbell has a number of practical advantages in diagnostic work It is very economical of serum and antigen and also of glassware Since the commercial virus antigens are rather expensive, the drop method has been popular in virus laboratories and has proven useful (Fulton, F and Dumbell, K R *J Gen Microbiol*, 3 97 111, 1940)

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This Book

INTRODUCTION TO VIROLOGY

By GILBERT DALLDORF, M D

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